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Control of Gene Expression

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Preface

Almost all animal and plant cells contain at least one and often two copies of each gene possessed by the whole organism. Realization of this astonishing fact, and of its consequences for genetics, has developed gradually over the last century. One implication of this broad gene distribution is that those few genes which are actively expressed in a cell at any one biological place and time must be preferentially selected for activity: meanwhile the vastly greater number of genes within that cell which are biologically irrelevant to its structure and function remain silent and unexpressed. Although we now have some inkling as to how such differential gene expression is accomplished in bacteria, we are largely ignorant of the process in eukaryotic organisms. What is clear, however, is that the process in higher cells is not itself simple, and that many complex mechanisms are likely to be involved.

This book does not, therefore, set out primarily to explain how gene expression is controlled in eukaryotes, but rather to discuss and compare the experimental systems currently being used to investigate this problem. I have attempted to sketch fairly briefly the present state of knowledge of gene expression in bacteria in Chapter 2 and the probable roles of the nucleic acids in the process in eukaryotes within the concluding chapters. However, the greater part of the book is devoted to a discussion of some of the more important or promising experimental systems currently being exploited in this area by biologists and biochemists. I hope the book will prove of use to students and scholars whether they are on the "inside" or the "outside" of these systems. Not only does the book indicate the "state of play" within the experimental systems at the moment, but it also attempts to compare present results and future potential. Here I tread on dangerous ground. Not only am I seriously limited by my own partial knowledge of some of these systems—with only one, the synthesis of haemoglobins, am I intimately concerned—but my own judgement about which systems deserve mention and which are the more promising is bound to be a very arbitrary one.

Writing the book has confirmed in my own mind however, a suspicion which I entertained at the outset. It is that many of those engaged in exploiting these systems in laboratories throughout the world are often poorly informed about the advances and advantages of other systems aiming at the same intellectual goals. When experiments are reported and discussed in the literature, the authors often fail to mention similar or contrasting evidence gleaned from another system. Such an omission may sometimes be intentional, but more often, in my view, it is accidental and simply reflects the near impossibility of constantly keeping abreast of the vast relevant literature. Perhaps this volume will help to partially alleviate that problem.

Because much of the book involves a discussion of experimental systems with which I am not in day to day contact, I have lent heavily on others with more intimate knowledge and "inside" understanding than myself. These people have generously given their time to read and criticize and I am very greatly indebted to them. They include:

Dr. Ruth M. Clayton
Prof. J. M. Mitchison
Prof. G. T. Stevenson
Dame Honor Fell
Dr. D. J. Cove
Dr. V. A. Hilder
Dr. W. T. Drabble
Dr. A. E. Wild
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Dr. J. R. Tata
Dr. J. B. Gurdon
Dr. W. R. Branch
Prof. F. C. Kafatos
Dr. G. Goldspink
Dr. D. A. Morris
Dr. J. M. Barry
Dr. D. R. Garrod

Of course I have not invariably taken their advice and in any event the opinions expressed in the book are my own unless I have indicated otherwise.

Many others have read and criticized small sections or provided invaluable discussion and contact. Most of the plates have been generously provided by other biologists both here and in the United States. These have been acknowledged within the body of the book. My own colleagues in the Department at Southampton have provided a friendly and stimulating environment for my own efforts, both scientific and literary, and the typing skills of Mrs. Anne Wharmby, Mrs. Mavis Lovell and Mrs. Monica Meek are gratefully acknowledged.

N. Maclean

Southampton, 1975

Contents

Preface v

Chapter 1. **The control of gene expression and its levels of action** 1

A. Control at the level of transcription 3
  1. Elimination or destruction of the gene 3
  2. DNA sequences which are normally incapable of transcription 3
  3. Control by large scale choromatin condensation 4
  4. Control of one sequence by another 5
  5. Effects of one gene on its homologue 6
  6. Specificity and availability of RNA polymerase enzyme 7
B. Experimental approaches to transcriptional regulation 7
  1. Antagonists of transcription 7
  2. Molecular hybridization 8
C. Control at a post-transcriptional level 10
  1. Attachment of poly A sequences to Hn RNA 10
  2. Size reduction of the Hn RNA 10
  3. Nuclear screening of RNA transcripts 10
  4. Formation of protein mRNA complexes 10
  5. Ribosomal translation of mRNA and possible selection 11
  6. Messenger RNA lifespan and degradation 11
  7. Availability of amino acids and transfer RNA 12
  8. Release of the polypeptide chain from the ribosome 12
  9. Association of sub-units in polymer proteins 12
  10. Post-translation modification of proteins 12
  11. Specific storage or degradation of protein 13
D. Experimental approaches to post-transcriptional regulation 13

Chapter 2. **Gene expression in prokaryotes** 14

A. Genetic organization in prokaryotes 15
B. Transcriptional control 16
C. Bacterial operons (negative control) 17
D. Positive control 19
E. Sequential control of gene expression 21
F. Multiple gene copies 23

G. Recognition of base sequences by tertiary structure . . . 24
H. Post transcriptional control . . . 24
I. Expression of extrachromosomal genetic factors in bacteria . . . 28
J. Comment . . . 29

Chapter 3. **Experimental systems of differential gene function in eukaryotes—systems involving one type of protein** . . . 30

A. Immunoglobulins . . . 30
  1. Gene clustering . . . 30
  2. Allelic exclusion . . . 36
  3. Preservation of allotypic identity with idiotypic variation . . . 37
  4. Somatic mutation . . . 38
  5. Translocation . . . 39
  6. Cellular control of antibody production . . . 40
  7. Comment . . . 41
B. Lactate dehydrogenase . . . 41
  1. LDH subunits with very restricted expression . . . 44
  2. The distribution of the A and B type LDH subunits . . . 45
  3. Different tissues of the adult animal display different LDH patterns . . . 46
  4. The same tissue in one animal may display different LDH patterns at different times in ontogeny . . . 51
  5. Specific degradation of LDH isoenzymes . . . 51
  6. Comment . . . 52
C. Haemoglobin . . . 53
  1. Structure and distribution of haemoglobin . . . 54
  2. Erythroid cells . . . 59
  3. Control of haemoglobin synthesis . . . 63
  4. The erythrocytes as a terminally differentiated cell . . . 71
  5. Erythrocyte included in fused hybrid cells . . . 71
  6. Haemoglobin synthesis by erythroleukaemic cells . . . 73
  7. Globin gene frequency in the genome . . . 74
  8. Comment . . . 74
D. Lens crystallins . . . 75
  1. Regulation of crystallin synthesis . . . 76
  2. Ontogenic succession of crystallins . . . 78
  3. The differential distribution of crystallins . . . 79
  4. Stability of messenger RNA . . . 79
  5. Other lens proteins . . . 80
  6. Lens regeneration . . . 81
  7. Lens fibre differentiation *in vitro* . . . 81
  8. Comment . . . 82
E. Milk proteins . . . 83
  1. Hormonal influences on mammary gland differentiation and synethesis . . . 85
  2. The insulin effect and cell division . . . 86
  3. The specific effect of prolactin . . . 87
  4. Genetic variants of milk proteins and mRNA isolation . . . 87

  5. Linkage of the milk protein genes . . . 88
  6. Comment . . . 88
F. Fibroin . . . 88
  1. Polyploidy of the silk glands . . . 89
  2. Transcriptional and translation control in fibroin synthesis . . . 90
  3. Isolation of fibroin messenger RNA . . . 92
  4. Comment . . . 92
G. Cocoonase . . . 93
  1. Chemical characteristics of the protein . . . 94
  2. Synthesis of the protein . . . 94
  3. Comment . . . 97
H. Vertebrate egg proteins . . . 97
  1. Egg yolk proteins . . . 98
  2. Egg white proteins . . . 100
  3. Comment . . . 102

Chapter 4. **Experimental systems of differential gene function in eukaryotes—systems of limited complexity** . . . 104

A. Pancreas . . . 104
  1. Exocrine pancreas . . . 105
  2. Endocrine pancreas . . . 107
  3. Comment . . . 110
B. Enzyme synthesis during the yeast cell cycle . . . 111
  1. Comment . . . 116
C. Muscle . . . 117
  1. Structure of skeletal muscle . . . 118
  2. The development of skeletal muscle in the embryo . . . 120
  3. Muscle cell proteins . . . 122
  4. Control of the levels of muscle proteins . . . 127
  5. Comment . . . 130
D. *Aspergillus* and *Neurospora* . . . 130
  1. Genome associated proteins . . . 131
  2. Positive control of fungal genes . . . 131
  3. Induction by product . . . 133
  4. Gene clustering . . . 133
  5. Comment . . . 135
E. Nuclear transplantation and microinjection . . . 136
  1. Nuclear transfer by micromanipulation . . . 137
  2. Nuclear transplantation and labelled molecules . . . 141
  3. Cell fusion experiments . . . 141
  4. Microinjection of cell contents other than nuclei . . . 145
  5. Microinjection of messenger RNA . . . 146
  6. Comment . . . 150
F. Cartilage . . . 151
  1. Differentiation of cartilage *in vitro* . . . 153
  2. Cartilage differentiation *in vivo* and in organ transplants . . . 155
  3. The effects of BUdR on cartilage differentiation . . . 155
  4. Comment . . . 157

G. Liver . . . 158
  1. Hormonal control of liver enzymes . . . 161
  2. Inducible enzyme synthesis in liver . . . 162
  3. Enzyme synthesis in amphibian liver . . . 163
  4. α Foetoprotein in liver . . . 164
  5. Comment . . . 165
H. DNA—associated proteins . . . 165
  1. Histones and other basic proteins . . . 166
  2. Histone structure and function . . . 167
  3. Histone genes . . . 170
  4. Structure of histone in chromatin . . . 172
  5. Non-basic proteins . . . 172
  6. DNA Polymerase . . . 172
  7. RNA Polymerase . . . 174
  8. Regulatory non-basic proteins . . . 176
  9. Reconstitution of eukaryotic chromatin . . . 181
  10. Comment . . . 183

Chapter 5. **Experimental systems of differential gene function in eukaryotes—systems not well understood in molecular terms** . . . 184

A. The early mammalian embryo . . . 184
  1. What determines cell fate in the mammalian embryo? . . . 184
  2. Are inner and outer cells different . . . 187
  3. Teratomas from embryonic cells . . . 188
  4. Comment . . . 190
B. Slime moulds . . . 192
  1. The cellular slime moulds . . . 192
  2. The plasmodial slime moulds . . . 197
  3. Comment . . . 200
C. Protozoa—studies on cortical inheritance . . . 200
  1. Cortical inheritance in *Paramecium*—cortical proteins . . . 200
  2. Cortical inheritance in *Paramecium* and *Stentor*—cortical pattern . . . 202
  3. Comment . . . 205
D. *Acetabularia* . . . 205
  1. Grafting procedure in Acetabularia . . . 208
  2. Nuclear transplants . . . 209
  3. Biochemical aspects of the *Acetabularia* experiments . . . 210
  4. The autonomy of the chloroplasts . . . 214
  5. Comment . . . 214
E. *Hydra* . . . 215
  1. Regeneration in explanted *Hydra* tissue . . . 217
  2. Comment . . . 220
F. The imaginal discs in *Drosophila* . . . 221
  1. The onset of developmental commitment . . . 224
  2. Homeotic mutants . . . 226
  3. Transdetermination . . . 229
  4. Phenocopies . . . 231
  5. Specification . . . 231

  6. Comment . . . 233
G. Higher Plants . . . 233
  1. Growth of plants from isolated somatic cells . . . 234
  2. Crown gall in plants . . . 234
  3. Phase growth in ivy (*Hedera helix*) . . . 236
  4. Unusual genetic elements in maize and *Antirrhinum* . . . 238
  5. Comment . . . 240
H. Mitochondria and chloroplasts . . . 241
  1. The petite mutant in yeast . . . 244
  2. Chloroplast genetics in *Chlamydomonas* . . . 246
  3. Comment . . . 248

Chapter 6. **RNA involvement in gene expression** . . . 250

A. Ribosomal RNA . . . 250
  1. Synthesis and processing of RNA . . . 250
  2. Function of the ribosome . . . 251
  3. Arrangement and expression of genes for ribosomal RNA . . . 252
  4. Amplification and magnification of genes for rRNA . . . 255
B. Transfer RNA . . . 257
C. Heterogeneous nuclear RNA . . . 258
D. Messenger RNA . . . 261
  1. Characteristics of the molecule . . . 261
  2. Synthesis and precursors . . . 262
  3. Utilization in the cytoplasm . . . 264

Chapter 7. **General concepts of gene regulation** . . . 266

A. Chromosome structure and gene masking . . . 266
  1. Inactivated blocks of genes . . . 267
  2. Repeated DNA sequences . . . 269
  3. Controlled transcription of single genes . . . 272
  4. The use of BUdR(5-Bromodeoxyuridine) . . . 276
  5. Integration of gene expression in differentiation . . . 276
B. Post-transcriptional control and the role of the cytoplasm . . . 279
  1. Regulation after translation . . . 280
  2. Modification of nuclear gene activity by the cytoplasm . . . 280
  3. Cytoplasmic DNA—mitochondria and chloroplasts . . . 281
C. Hormones and other extracellular factors . . . 283
  1. Animal hormones . . . 283
  2. Plant hormones . . . 286
  3. Chalones . . . 287
  4. Phytohaemagluttinin . . . 288

References . . . 289
Additional references . . . 320
Author Index . . . 323
Subject Index . . . 336

To Jean
and to all at
Pitmurthly

1. The Control of Gene Expression and Its Levels of Action

A. Control at the level of transcription . . . 3
B. Experimental approaches to transcriptional regulation . . . 7
C. Control at a post-transcriptional level . . . 10
D. Experimental approaches to post-transcriptional regulation . . . 13

In the broadest terms an animal or plant is the result of an interaction between its genetic material and the environment. Studies on gene expression may therefore, in the widest sense, include work on the yields of vegetable crops, the behaviour of octopuses or the development of fish. This book interprets gene expression in a more limited but fundamental way. We will here concentrate on the synthesis of particular proteins as the expression of individual genes, only occasionally discussing the later modification of the protein molecules and rarely emphasizing the function of the proteins as the determinants of the ultimate animal or plant form.

Even if gene expression is accepted in the limited sense, as being the production of particular protein molecules, this production line still involves a number of different levels of control. Control at the level of transcription of the gene sequence into RNA is exerted by the gene sequence itself, sometimes also by neighbouring sequences, by the state of the chromatin in which the gene is located, by the availability and specificity of the RNA polymerase enzymes and by the availability of the appropriate nucleotides for synthesis of that RNA. For those genes whose expression involves synthesis of protein by translation of the RNA intermediate (omitting, that is, genes coding for RNA and not for protein, e.g. ribosomal RNA genes) a whole spectrum of post transcriptional control mechanisms exists, involving modification of the transcribed RNA precursor, availability of functional ribosomes and the regulated destruction of the messenger RNA molecule.

A fundamental consideration in any research is that no single experimental system can be expected to ideally or even usefully reveal information on all aspects of a problem. When the problem is as broad as the control of gene expression this limitation is all the more evident. It follows that a wide variety of different experimental systems must be exploited in order to illuminate different aspects, the choice of experimental system being of paramount importance since it, more perhaps than the methodology or the insight of the investigator, determines what can and cannot be achieved. Although there is a tendency for the investigator to favour his system as a useful model for all aspects of the problem, an objective view soon betrays the limitations of most systems and the particular advantages of the best.

Any comparison of different experimental approaches to the same problem or to related problems exposes some other important observations. One is that, not infrequently, workers in one experimental area do not refer in their publications to equally or more informative results arising from a different but related experimental system. Sometimes this omission may be deliberate, but, often it seems to be accidental and to follow from a real ignorance of what has been achieved in the other system. Another is that semantic barriers often seem to prevent cross-fertilization and understanding between two different but fundamentally related fields. One man's protein is another man's antigen. A "determined" cell to the embryologist may be a "committed" cell to the immunologist. A third observation is that, although words such as "control" and "regulation" are used (all too often to give a modern ring to an old idea) with increasing frequency, very little of the work involves controlled degradation. Synthesis is regarded as being more fundamental and precise than degradation, and few people cite the breakdown of intermediates or products as fundamental limiting mechanisms. But, in fact, any study of control of populations size and character, which emphasized birth and ignored death, would be plainly stupid. Most studies on molecular aspects of gene expression are essentially similar to population studies on animal or plant species, the effective population is a result of a temporary balance between birth rate and death rate, and between synthesis and degradation.

In this chapter we will list the most important (but by no means all) stages and mechanisms of control, moving sequentially from the gene sequence on the DNA to the functional protein. In some cases the protein product cannot be directly assayed but some indication of its presence or activity is monitored, as, for example, in cell surface properties. These will be included, but the general area of utilization and breakdown of the proteins themselves will be largely ignored, since this involves the broader areas of gene expression which ultimately combine to form the phenotype of the individual organism. In addition to noting the experimental systems which have contributed, or are likely to contribute, most to our knowledge of particular steps in the control sequence, some discussion of the available techniques and possible approaches to the various problems will be included.

A. Control at the level of transcription

1. Elimination or destruction of the gene

Very few organisms resort to these processes as a means of controlling gene transcription. Gall midges, *Cecidomyidae*, and the nematode worm, *Parascaris equorum*, both eliminate part of the total genome from the somatic cells (discussed in Gurdon and Woodland, 1968) and tetraploid cells tend to lose chromosomes as cell divisions proceed (Migeon and Miller, 1968). It is arguable, however, whether these devices are really aimed at silencing the expression of the genes on these eliminated chromosomes, or more simply at reducing the overall DNA content of the cell.

2. DNA sequences which are normally incapable of transcription

Some DNA, particularly the centromeric chromatin of most chromosomes, consists of simple sequence DNA (highly repeated short sequences), as discussed in Section 7A. Such DNA is often termed consititutive heterochromatin. Although such DNA is obviously amenable to replication, it is not normally transcribed, i.e. it is not transcribed by the endogenous polymerases of that cell, nor is it necessarily transcribed by bacterial polymerases *in vitro*, i.e. in some *in vitro* systems it is transcribed, in others it is not.

There are other tracts of DNA which are not normally transcribed *in vivo* by endogenous polymerase, but which can be readily transcribed by bacterial polymerase. These include the spacer DNA which links the ribosomal gene sequences and also the L strand of the DNA double helix—only one of the two strands of the DNA duplex is normally transcribed and acts as functional genetic coding material. This sense strand is termed the H-strand. Evidence for transcription of spacer and L strand

DNA comes from the work of Reeder and Roeder (1972) and Honjo and Reeder (1973). Presumably, the *E. coli* polymerase used in these experiments is not sufficiently restricted in its activity and commences transcription at sequences which are not true initiator sequences. It also seems possible that the interchromomeric DNA of the polytene and lampbrush (and perhaps other) chromosomes falls into this transcriptional category of spacer DNA (see page 267).

3. Control by large scale chromatin condensation

As discussed more fully in Section 7A, large tracts of chromatin, and often entire chromosomes, are inactivated in particular cells by being rendered heterochromatic—faculative heterochromatin in this case. Thus, the transcriptional inactivity of the heterochromatic X chromosome in the human female, the Barr body of somatic female nuclei. Many other examples are known, including the heterochromatization of the entire parental set of chromosomes in the male scale insect (Brown and Nur, 1964). The condensation and heterochromatic character of the Y chromosome in the male mammal is a slightly different phenomenon and may be termed semifacultative heterochromatin (see Section 7A). Much, but not all, of the chromatin of this chromosome consists of simple sequence DNA.

We must also include here the striking examples of position effect, in which genes translocated to the vicinity of a block of heterochromatin have their genetic expression modified (Section 7A) or silenced. Such heterochromatization appears to block the RNA polymerase enzymes and to involve neighbouring chromatin in this impediment. As is discussed on page 271, although constitutive heterochromatin is structurally distinct from other chromatin, the differences between facultative heterochromatin and euchromatin are not dramatic in terms of composition, and are apparently mainly explained by an altered packing arrangement.

Most inactive chromatin is not, strictly speaking, heterochromatin, however. The condensed DNA of the amphibian erythrocyte nucleus, the mitotic chromosome and the unpuffed chromomeric band of the *Drosophila* polytene chromosome do not fulfil the normal requirements of heterochromatin, but their transcriptional inactivity is surely correlated with their condensation. Whether condensed euchromatin is actually analogous to facultative heterochromatin in its packing arrangement, or whether some crucial factor renders the one more permanent than the other, is not at present clear. But all three systems mentioned, the nucleated erythrocyte, the mitotic chromosome and the polytene chromosome chromomere, constitute valuable experimental systems for the study of transcriptional regulation.

4. Control of one sequence by another

This is no doubt the chief means of regulating the activity of most of the genetic activity, both in prokaryotes and eukaryotes. Whereas in the former the control gene sequences are few, and may well account for less of the entire genome than do the structural genes themselves, it seems likely that in eukaryotes most of the genome is given over to control, and each structural gene sequence is probably coupled with (and probably preceded by) a long tract of control gene sequences. Of course, the simplest type of transcriptional system is the one which involves no control and all genes are transcribed maximally all the time. With the exception of some simple viruses (see Chapter 2) this situation does not normally occur in living organisms. Instead, although some genes such as those coding for 18 and 28S ribosomal RNA may indeed be uncontrolled and except in very condensed chromatin will always be available for transcription, most genes are regulated in their activity. Such regulation is mediated either by regulatory molecules or controlled chromosome condensation, or both, and these aspects are discussed in Chapters 6 and 7.

Another possible mechanism for transcriptional regulation is linear reading, in which genes would be transcribed in the order of their arrangement on the chromosome, and their position would be tightly correlated with the time in the cell cycle when their activity was required. Provided that messenger RNA half life is not too short, such a system can presumably operate effectively and some slight evidence favouring such a system has been found in studies on the yeast cell cycle (see Chapter 4B).

The exciting growth of knowledge about bacterial operons and regulons has stimulated an intensive search for similar genetic control mechanisms in eukaryotes, but so far without success. One group of genes in the fungus *Aspergillus* is expressed coordinately in a manner suggesting a rather loose operon, but all other genes in eukaryotes, whether linked or unlinked, appear to be controlled singly. Since both positive and negative control of bacterial operons is known, attempts have also been made to identify the type of control prevalent or universal to eukaryotes. The evidence from fungi (see Section 4D) suggests positive control and many theories of eukaryotic gene regulation are based on this model. However, as discussed in Section 7A, the negative control mechanism may well be more typical, in view of the apparent transcription of the control sequences in the formation of the Hn RNA as precursor to mRNA.

5. Effects of one gene on its homologue

Most eukaryotes are diploid and two copies of each gene are normally present in every cell, either as identical or non-identical alleles. Presumably the frequent phenotypic effect of gene dominance or partial dominance is not normally a result of transcriptional control but of competition between differing forms of the same protein for functional or structural priority.

In the case of the facultative heterochromatization of one of the two chromosomes in the human female X or the male mealy bug, only one of the two gene copies is expressed, but the transcribed gene copy may differ from one cell to another, depending on which chromosome happened to be condensed. But two other examples are known which are not believed to be explained by condensation of one of the two sister chromosomes involved. The first is the "allelic exclusion" phenomenon demonstrated by the pattern of synthesis of immunoglobulins (see Section 3A). No mechanism is known which can account for this remarkable phenomenon, but presumably the product of one of the two alleles automatically inactivates the other copy, perhaps depending simply on which copy happened to be first transcribed. The second example is revealed by studies on the types of ribosomal RNA synthesized during the development of *Xenopus laevis × mulleri* hybrids (Honjo and Reeder, 1973). The ribosomal RNAs (18 + 28S) of the two species do not in fact vary, but the precursor RNA molecules can be readily distinguished by hybridization since the spacer sequences do differ considerably. Hybrids synthesize only the *laevis* type, irrespective of which species is the male and which is the female in the cross. Now it may be that this curious effect can be explained either by the RNA polymerase of the one species blocking the promoter or control sites on the homologous ribosomal RNA genes (a special form of RNA polymerase is responsible for transcription of the ribosomal sequences) or, as suggested for allelic exclusion in immunoglobulins, that the transcriptional product of one gene (or its near neighbouring sequences) effectively represses transcription of the rival sequences in the hybrid.

6. Specificity and availability of RNA polymerase enzyme

As is discussed in Chapter 2, the main RNA polymerase enzyme of bacteria can be split into core and sigma fractions, the latter subunit having the ability to restrict preferentially the affinity of the enzyme for certain promoter gene sequences. No evidence has so far been obtained for a similar restriction operating in eukaryotic cells. But this is not to say that no such mechanism exists. Many genes which are transcribed seem to be much more actively transcribed at certain parts of the cell cycle than others, or in some cells than others. Work on the yeast cell cycle (Section 4B) and on the expression of the lactate dehydrogenase genes (Section 3 B) suggests that rate control is exerted over the transcriptional activity of certain genes. Probably the simplest mechanism for effecting such rate control would be via the affinity of the RNA polymerase for the promoter sequence. Of course other forms of control might easily operate, especially some form of end-product inhibition of the specific sequence transcription. Again, the best examples of such mechanisms are to be found in fungal systems (Section 4D). The existence of separate eukaryotic RNA polymerases for the ribosomal and other sequences, presumably, permits rather distinct kinds of control to affect these two types of sequence.

B. Experimental approaches to transcriptional regulation

1. Antagonists of transcription

Amongst the wide range of antibiotic substances now known, several have important and specific effects on transcription and provide the investigator with extremely valuable tools. Perhaps the best and most widely used is Actinomycin D, which binds to DNA and effectively prevents transcription. Some variation in effect is noticeable in different cells and organisms, probably due to variable permeability of the cells to the drug. An excellent review of the mechanism of action of Actinomycin D and other antibiotic drugs can be found in Gale (1972). Actinomycin will, at high doses, effectively inhibit all RNA polymerase activity, but at doses below one μg/ml it becomes relatively specific for the synthesis of ribosomal RNA and at higher doses for messenger RNA. The use of actinomycin D in the experimental work on *Acetabularia* has been particularly valuable and is discussed in the appropriate chapter.

A second antibiotic, Rifampicin, is a semisynthetic derivative of the naturally occurring rifamycin. This drug is a potent inhibitor of bacterial DNA-dependent RNA polymerase, but not of the eukaryotic RNA polymerases, except probably those of mitochondria and chloroplasts (the impermeability of mitochondria to the drug renders it ineffective *in vivo*). There is also some evidence that rifampicin will inhibit some viral polymerases and so may have value as an antiviral drug. A third substance α-Amanatin, which occurs naturally in the fungus *Amanita phalloides*, is valuable in a way complementing the use of rifampicin. γ-Amanatin inhibits only the non-nucleolar RNA polymerase of eukaryotic cells, leaving bacterial, mitochondrial and nucleolar (ribosomal) RNA synthesis mainly unaffected. Although we cannot here discuss the very numerous experiments undertaken with these inhibitors of nucleic acid synthesis, various projects discussed in later chapters in this book involve the use of these valuable drugs.

2. Molecular hybridization

The discovery that single stranded nucleic acids will, under appropriate conditions, hybridize with another molecule of complementary base sequence, has proved of great experimental value in this area. Not only may DNA to DNA and DNA to RNA hybrids be monitored, but the radio labelling of one of the hybrid monomers permits it to be used as a probe for the detection of complementary sequences within a cell or cell extract. In addition, with the recent availability or relatively pure bulk fractions of particular messenger RNAs and of viral reverse-transcriptase polymerases, it has become possible to synthesize radioactively labelled DNA probes which are complementary to known messenger sequences, thus permitting assay for such messenger RNA sequences in other cells and tissues. While applauding this advance we would do well to emphasize that the formation of such probes is dependent on the availability of relatively pure fractions of individual mRNA species, a fact which even in the next few years will surely limit such methods to less than a dozen different species of mRNA. So far the method has been employed in only a few examples, of which two may be mentioned, the first globin messenger RNA (Chan *et al.*, 1974 and Ross *et al.*, 1974) and the second ovalbumin messenger RNA (Cox *et al.*, 1974).

Having pointed out the limited extent of this valuable technique for the detection of specific mRNA molecules, it is also important to stress that this is the only direct approach to the study of gene regulation at the transcriptional level. The wealth of experimental data reviewed in later sections of this book consists almost entirely of observations of the synthesis or activity of protein molecules. The transcriptional events, which after all are those of primary gene regulation, can only be inferred from such observations, and very often such inferences involve numerous assumptions. In other words, our ability to study gene regulation at the basic transcriptional level is limited to the genes coding for transfer and ribosomal RNA and those few other genes that code for proteins which are made in circumstances which permit isolation of the relevant mRNA.

The aspects of genetic regulation which have been investigated by molecular hybridization include the nature and distribution of DNA sequences on the genome (see Section 7A), the nature and distribution of messenger and other sequences in the Hn RNA (see Section 6C), the production and utilization of transfer and ribosomal RNA and of a few species of specific messenger RNA.

To date, the really key question about control of transcription of structural genes remains unanswered, but the evidence does point towards an answer. That is, it is still not possible to be certain whether most eukaryotic transcriptional control is absolute so that proteins undetected in the cell are paralleled by genes totally inactive in the genome. Cox *et al.* (1974) have probably driven as close to a complete answer as any finding that, prior to normal induction, the cell which is capable of synthesizing ovalbumin contains, on average, 1 molecule of ovalbumin mRNA only. Following normal induction the level rises to as many as 36 000 mRNA molecules per cell. Such evidence, interpreted with great caution by the authors, does strongly suggest a total repression of the gene sequence prior to induction, followed by specific gene unmasking after hormonal induction (see further discussion in Section 3H).

C. Control at a post-transcriptional level

As discussed in Section 6C, the primary transcriptional product of most eukaryotic genetic units is a relatively high molecular weight RNA (Hn RNA). The ribosomal cistrons also produce a large primary transcript. The first stage of post transcriptional control is, therefore, the processing of this transcript.

1. Attachment of poly A sequences to Hn RNA

As discussed in Section 6C, many, but not all, of the Hn RNA molecules receive a tail of some 200 adenine residues at their 3′ terminus. These sequences persist when the Hn RNA is cut down to size to form the messenger RNA, and there is some evidence from the globin synthesis system that the poly A sequences may help to determine the life span of the mRNA molecule.

2. Size reduction of the Hn RNA

Preparations of RNA are bedevilled on the one hand by fears of aggregation artefacts and on the other hand by fears of degradation artefacts. Both aggregation and degradation are speedy processes and the validity of any RNA preparation must always be considered in the light of these hazards. Some very large Hn RNA molecules recovered from nuclei may, therefore, be aggregation artefacts, but a fairly large fraction of molecular weights between one and ten million can be isolated. Messenger RNA is probably rarely more than 1000 daltons in weight, indicating that a considerable size reduction in the Hn RNA occurs in the nucleus.

3. Nuclear screening of RNA transcripts

It has often been suggested that the nucleus might sieve the products of the active genes and so control the amounts and types of mRNA permitted access to the cytoplasm. This notion remains entirely without experimental support, since the population of Hn RNA molecules which fail to receive poly A sequences also appear to lack messenger sequences (Nakazato *et al.*, 1974).

4. Formation of protein mRNA complexes

It is not clear whether mRNA leaves the nucleus as an isolated molecule or already coupled with protein, but certainly cytoplasmic mRNA is either found bound to ribosomes or to one or more protein molecules. It is difficult to discriminate between ribosomal proteins which might remain bound to detached mRNA, and specific non-ribosomal proteins bound to mRNA to form a ribonucleo-protein particle (RNP). Such particles have been isolated from erythroid cells (see Section 3C) and may well be a stabilized form of most or all classes of mRNA. Conjugation with protein may also constitute an important mechanism of mRNA storage or sequestration. Stored non-functional mRNA is apparently present in unfertilized eggs (where the RNP particles have been termed informosomes by Spirin (1969)) and in *Acetabularia* (see Section 5D).

5. Ribosomal translation of mRNA and possible selection

The cell free system has been extensively exploited to reveal the facts about eukaryotic translation in protein synthesis, the reticulocyte system proving particularly useful (see Section 3C). There is as yet no really reliable evidence that ribosomes select amongst the population of available mRNA, and the mRNA injection experiments of Gurdon (1974) (discussed in Section 4E) argue against such phenomena. Modification of the mRNA or its protein product might, of course, occur on the ribosome and this is discussed in prokaryote systems in Chapter 2. Many of the experimental systems discussed later in this book provide examples of dramatic changes in the total ribosome population in answer to new and special needs of the cell during its differentiation. The special properties or functions of free ribosomes, and those bound to the endoplasmic reticulum, are discussed in Section 6A and remain problematical.

6. Messenger RNA lifespan and degradation

Whether or not mRNA lifespan is truly governed by its tail of poly A sequences remains uncertain, but there is ample evidence for varying half-lives of different mRNA within one cell and for the same species of mRNA in different cells. mRNA lifespan, even in eukaryotes, seems to vary between minutes and months and clearly most are translated, either by the same or a different group of ribosomes, many times. It is easy to believe that the controlled degradation of different mRNA molecules, either by planned obsolescence of the mRNA or by special ribonucleases, must consitute an important device in the control of protein synthesis, but this aspect of control has attracted little experimental attention and remains speculative. Specific stabilization of mRNA is often cited as a control mechanism in cell differentiation, e.g. in lens cells synthesizing crystallin and erythroid cells synthesizing globin.

7. Availability of amino acids and transfer RNA

Shortage in the supply or availability of one or more amino acids could affect the types of protein being synthesized. As discussed in Section 6B, some cells do come to possess an unusual distribution of tRNA molecules but whether this is truly designed to control protein synthesis is uncertain.

8. Release of the polypeptide chain from the ribosome

The rate of protein synthesis is partly affected by the release of the polypeptide chain and some polysomes can apparently remain blocked by completed chains. Discussion in Section 3C of the influence of free α globin chains on the release of other α globin and also on β globin chain release is probably the best example of actual control over release from the ribosome.

9. Association of subunits in polymer proteins

Many structural and enzymic proteins are dimers or tetramers and may not function in a monomeric form. Moreover, in the case of many isoenzymes, e.g. lactate dehydrogenase, more than one monomer type is synthesized, permitting different tetramers to form by differing monomer association. Lactate dehydrogenase (Section 3B) illustrates very well the marked influence of the intracellular milieu on which monomers are available and which polymers persist as stable functional proteins.

10. Post-translational modification of proteins

Many proteins are first synthesized as a zymogen and only become active following a post translational cleavage or trimming mechanism. The egg yolk protein vitellogenin (see Section 3H) and the cocoonase enzyme of silk moths (see Section 3G) present interesting examples of this important process. Other proteins may be acetylated or methylated after synthesis and these processes seem to be particularly important in the case of the nuclear histone and non-histone proteins (see Section 4H).

11. Specific storage or degradation of protein

The relative accessibility of protein to degradative processes is one of the key aspects of the control of gene expression. During the cell cycle or during tissue differentiation, some proteins are degraded to make way for others, either the same or different in composition. Proteins which are preserved, e.g. the lens crystallins, are probably highly exceptional and the interesting variations in life span of LDH enzymes in different tissues (see Section 3B) are almost certainly more characteristic. Some interesting reviews have recently appeared on protein degradation and its specificity (Schimke, 1973; Goldberg and Dice, 1974). The main processes of protein utilization in the life of the cell take us into the more distant aspects of gene expression however, which, though no less important, are beyond the scope of this book.

D. Experimental approaches to post-transcriptional regulation

Many techniques are utilized for studies on this level of genetic regulation in cells, and we cannot attempt here to survey them. But one or two important points can be made. The first is that inferences about genetic transcriptional control should not be made too hastily from studies on the protein product, or even of the mRNA in the cytoplasm. It is tantamount to determining the order in which handicapped runners started a race by watching the middle or finishing stage of the race. Secondly, it is vital to distinguish between studies which monitor the presence and quantity of a protein, say by immunofluorescent techniques or polyacrylamide gel separation from those which measure protein activity, say by enzyme assays. The latter are at a stage later in the race than the former. Measurements of protein or RNA availability must also be seen as a balance between synthesis and degradation, often complicated by precursor pools and likely to fluctuate greatly in some tissues at different times.

But having emphasized the necessity for caution or scepticism, it is only fair to state that many experimental systems, some essentially molecular and others truly whole-organism studies, have made exciting contributions to our knowledge of gene expression in recent years. We will now proceed to look in greater detail at some of the best or the most promising.

2. Gene Expression in Prokaryotes

A. Genetic organization in prokaryotes 15
B. Transcriptional control 16
C. Bacterial operons (negative control) 17
D. Positive control 19
E. Sequential control of gene expression 21
F. Multiple gene copies 23
G. Recognition of base sequences by tertiary structure 24
H. Post transcriptional control 24
I. Expression of extrachromosomal genetic factors in bacteria 28
J. Comment 29

Prokaryotes are organisms which lack discrete nuclei and include bacteria, blue green algae and mycoplasma. Although viruses do not boast a cellular organization, they do have a genetic structure and are capable of replication within a cell. It is, therefore, appropriate to include viral genetic expression within the context of this chapter.

One of the most astonishing results of scientific advance over the last decade has been that, while considerable ignorance still prevails in the area of human genetics and control of human gene expression, and for that matter about eukaryotic genetics in general, knowledge of bacterial and viral genetics is now extensive. Indeed, for one of the smallest of the viruses, the bacteriophage lambda, knowledge promises to be complete within the next few years. This state of affairs follows closely from the comparative ease of doing genetic experiments with bacteria and viruses.

Prokaryotes possess many advantages in terms of simplicity of genetic organization and control. Compared to the multiple chromosomes of eukaryotes, bacteria and viruses have only a single genetic unit or chromosome, and in the cellular organization of this unit, the genetic material is not conjugated to protein. The histones of eukaryotic chromosomes serve to mask their genetic material as much and perhaps more from the microscopist as they do from the attention of polymerase enzymes, while the availability of prokaryotic genetic material makes for a simplification of experimental detection and study. The genetic availability of the prokaryotic genome does not only extend to lack of chromosomal protein, for the lack of a nuclear membrane between genome and cytoplasm also ensures that the genetic material is always available to the cytoplasmic molecules.

The lack of a nuclear membrane in the prokaryotic organization is possibly the main reason why it is so much easier to study and to understand gene expression in prokaryotes than in eukaryotes. In the prokaryotic cell, whenever a molecule is released from the DNA, it is already in the cytoplasm. Indeed, as is visualized in some of the photographs of Dr. Oscar Miller, ribosomes are already engaged in translating an RNA molecule in the bacterial cell before it has become completely detached from the DNA. Translation begins before transcription ends. As has been discussed in Chapter 1, very great difficulties attend the study of eukaryotic gene expression at the level of the protein product. This is because many levels of control intervene between transcription and translation, many genes may be transcribed which are never translated and other genes may be translated into detectable protein long after their active transcription has ceased. This objection is much less serious with bacteria and viruses, partly because of their rapid growth rate, and it is here much more justifiable to determine which genes are being transcribed at any particular time by analysing the proteins being synthesized. Until such times as different species of messenger RNAs can be identified from one another, advances will remain very dependent on the method of scrutinizing the protein in order to determine which genes are active and when.

A. Genetic organization in prokaryotes

The size of the entire genetic unit of viruses varies from about 3000 nucleotides in some very small RNA virus, through about 45 000 pairs in phage lambda, to some 200 000 base pairs in T even phages. If a gene is taken to consist of at least 1000 nucleotide pairs, then it follows that lambda will have about 45 genes and T_2 about 200. The bacterium *E. coli* has a chromosome made up of rather more than four million base pairs, suggesting a total gene complement of not more than 4000. This figure may be high, since an average protein molecule with about 500 amino acids would require 1500 nucleotide pairs, but regulatory genes are probably smaller. At any event, the prokatyotic genome consists of an organization of up to 4000 genes making up a haploid DNA amount of 0·0025 picograms (actually many bacteria have more than one chromosome per cell in growth phase because DNA replication can outstrip cell division but cells are reduced to the haploid amount at division). Since eukaryotic cells have anything from 20 to 20 000 times as much, it is plain that prokaryotic genetics is, by and large, much less complex than eukaryotic genetics. If this is so far the overall numbers of genes, it is likely to be even more so for the particular concern of this chapter, the control of gene expression.

Analysis of the nature of the gene and its fine structure has been particularly fruitful in bacteria and viruses and the penetrating experiments of Benzer (1961) have revealed that, for two genes of the bacteriophage T_2, the mutable site is within the gene and is indeed the nucleotide itself. The fact that the nucleotide sequence of the gene is transcribed and translated in a strictly linear manner has also been demonstrated by the classic work of Crick and Brenner (1967) and Yanofsky *et al.* (1967) on phage bacterial systems.

So the prokaryote genome consists of some thousands of genes, each made up essentially of a string of 1000 or so nucleotides, and being transcribed normally from end to end. How is the expression of such a unit controlled? This question makes an important assumption which deserves attention. Is there any control? The simplest form of genetic expression would be to have all genes available for transcription and translation all of the time. Is there anything to suggest that prokaryotes do control their genetic expression? The answer is yes, even for simple viruses (As discussed later in this chapter, single stranded DNA phages may lack even this sequential control of expression). It is possible to generalize with reasonable confidence that the prokaryotic genome is not all expressed all of the time (it is hard to rule out the possibility of expression, i.e. transcription and translation, operating for some genes at a level below our present limits of detection).

Having established that control is effective, how is it engineered? This will be discussed at the levels at which it is operative-transcriptional and post transcriptional.

B. Transcriptional control

Transcriptional control is control over the immediate products of transcription, namely RNA. The first observation on prokaryotic transcriptional control must be that many of the devices employed by eukaryotic organisms do not occur in prokaryotes, for example: elimination of whole chromosomes or parts of chromosomes from the cell, the long term repression of large chunks of chromatin in the form of permanent heterochromatin (e.g. Barr bodies and Y chromosomes), the severe condensation of chromatin found in the nuclei of many inactive cells by supercoiling of the nucleoprotein complex and gene amplification as found in the ribosomal RNA cistrons of some oocytes. None of these special devices of major transcriptional control occur in prokaryotes. Instead bacteria have developed a system of co-ordinate gene expression by which groups of genes coding for proteins with interrelated functions (assuming that interrelation of function requires that such proteins should all be available in the cell at the same time) are all controlled as a transcriptional unit. The evidence for such functional units of transcription is now a matter of scientific history and stems mainly from the ideas of Jacob and Monod, based on the phenomenon of induced enzyme synthesis. It is not appropriate to review here all the evidence which points towards such a regulatory mechanism, but an outline of the basic operation of an operon and the types of operon which are known will be necessary.

C. Bacterial operons (negative control)

An operon is a sequence of genes situated together on the DNA the transcription of which is controlled co-ordinately.

Basically, two kinds of operon exist in bacteria, one acting by negative control, the other by positive control. The type best known and first investigated by Jacob and Monod functions by negative control and such operons control the production of enzymes affecting, for example, lactose metabolism in *E. coli* and histidine synthesis in *Salmonella typhimurium* (Brenner and Ames, 1971). Present understanding of the lactose operon in *E. coli* indicates that it consists of a row of five genes arranged in the linear order, P—O—Z—Y—A:

P = promoter gene
O = operator gene
Z = β-galactosidase enzyme structural gene
Y = β-galactosidase permease structural gene
A = β-galactosidase transacetylase structural gene

A sixth gene, I, the regulator gene, is situated close to if not contiguous with P.

In the uninduced state, the repressor (the product of the regulator gene, I) occupies a site on the operator gene, O, and prevents transcription of the operon. When inducer molecules are available to the cell, e.g. lactose, they combine with the repressor molecules and, probably by an allosteric effect, prevent their effective repression of the operator gene. The operon is then in the induced state and the operon is transcribed. The promoter gene, P, has posed some problems for microbial geneticists. This gene, of about one hundred nucleotides in length, functions by initiating the transcription of the DNA into RNA by the polymerase enzyme and is strongly sensitive to levels of cyclic-AMP. Mutants of this gene may, therefore, be fully repressed due to lack of transcriptional initiation. Prior to 1968 the site of the P gene was believed to lie between O and Z, but it now seems certain (Ippen *et al.*, 1968) that P precedes O in the operon sequences. Certainly the evidence available points towards the operon being transcribed as a transcription unit into one polycistronic messenger. This can be deduced from the fact that induction of the *lac* operon leads to the appearance of a novel 30S RNA in *E. coli* which could conveniently code for all the cistrons on the operon and is certainly too large for the messenger RNA of the β-galactosidase enzyme alone (Guttman and Novick, 1963).

Recent facts relevant to negatively controlled operons can be summarized as follows:

(a) The repressor molecule has been recovered from operator genes and has been identified as a protein in the cases of the *lac* operon (Gilbert and Müller-Hill, 1966) and is a tetrameric molecule of about 7S, having a molecular weight of 150 000 (Riggs *et al.*, 1968) and comprising four identical subunits.

(b) Repressor molecules display properties suggestive of an allosteric protein (an allosteric molecule has at least two active sites and binding of a molecule to one active site induces a change in shape which alters the characteristics and binding properties of the second active site) since inducer molecules, such as IPTG, alter the affinity of the repressor for the operator gene (Zubay and Chambers, 1971).

(c) About 5% of the fully induced level of galactosidase enzyme is made in the complete absence of cyclic AMP, apparently by initiation of RNA polymerase activity at points other than the promoter gene. The promoter gene gives rate control over synthesis by promoting initiation of transcription in the presence of cyclic AMP (Zubay and Chambers, 1971).

Many other details and complications concerning initiation and regulation are known but cannot be discussed within the space available here.

D. Positive control

The first operons to be discovered in bacteria operated by negative control, that is to say that the operon was repressed by the product of the regulator gene occupying the operator gene site and induction involved derepression by removal of the repressor molecule from that site. However, it soon became clear that not all operons worked in this way and some, notably the genes concerned with L-arabinose synthesis in *E. coli*, almost certainly operate by a positive control (Englesberg, 1971). Other operons for which positive control is the likely mechanism are L-rhamnose metabolism (Power, 1967) and alkaline phosphatase (Garen and Echols, 1962), both in *E. coli.* In these positively controlled operons, the sequence at the controlling sites fails to facilitate transcription of the operon in the absence of the products of the regulatory gene. However, although the L-arabinase operon is positive in that sense, it also possesses some elements of negative control within it.

The L-arabinose operon consists of a row of three structural genes, gene A coding for L-arabinose isomerase, gene B for L-ribulokinase and gene D for L-ribulose 5-phosphate 4 epimerase. As shown in Fig. 1, these genes are arranged in the order D—A—B, with gene I, an initiator gene, and gene O, the operator gene, following. A sixth gene, C, the regulator gene, is situated slightly distant to the operator gene. Recent evidence indicates that the product of the regulatory gene C is a protein which displays aspects of alloteny, that is that can act as either repressor or activator depending on whether it is bound to the inducer L-arabinose. In the absence of L-arabinose, the regulator protein acts as a repressor and occupies the operator gene in that capacity. However, if L-arabinose is present, the regulatory protein becomes an activator molecule and, in this form and capacity, attaches to the initiator site rather than to the operator site. The absence of the repressor protein from the operator is a necessary condition for operon activity but maximum expression of the gene complex is dependent also on the altered form of the regulatory protein actually occupying the initiator site. Gene I, the initiator gene, has characteristics resembling the promoter

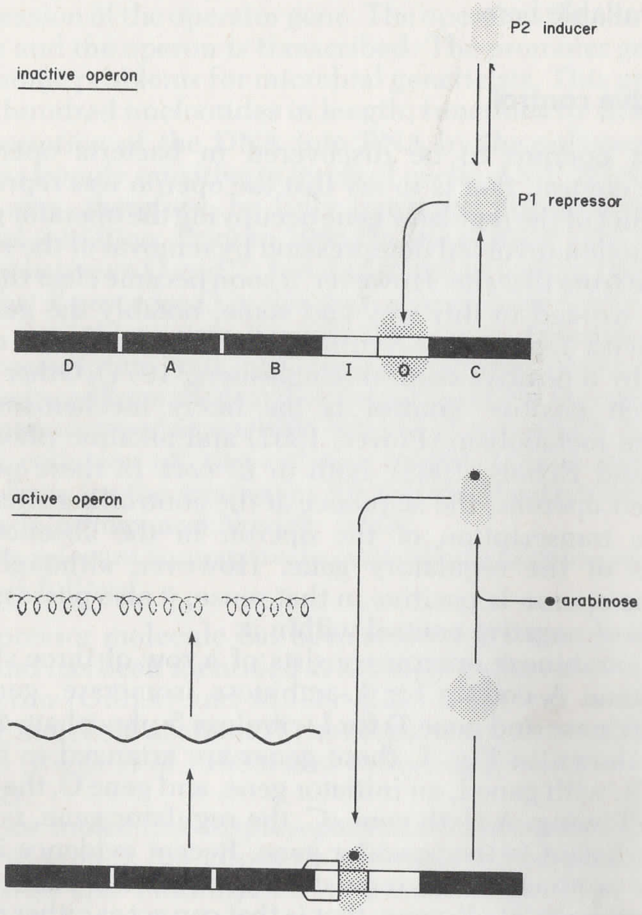


FIG. 1. Repressor-inducer model for control of the arabinose operon. The regulator gene araC codes for a repressor protein, Pl, which binds at the operator (ara0) to switch off the operon. The repressor is in equilibrium with an alternative protein conformation, P2, which acts as an inducer; but in the absence of arabinose Pl is the predominant state so the operon is inactive (upper). Addition of arabinose converts Pl repressor to P2 inducer; the inducer acts at the initiator site araI to switch on the operon. The molecular basis for its interaction is not defined but it is shown here as interacting with RNA polymerase to assist the initiation of transcription. (Model of Englesberg *et al.*, 1969. Reproduced with permission from Lewin, 1974a.)

gene of the *lac* operon but it cannot be so designated until it proves to be the site for initiation of mRNA synthesis.

Another interesting complication of L-arabinose metabolism and its control in *E. coli* is that the regulatory gene C also regulates an L-arabinose transport system co-ordinately with the O1BA D operon described above, and at least one of the enzymes for this transport system seems to be coded in a locus distant from the main operon. There is, therefore, some suggestion that the regulatory gene controls at least two separate operons, both involved in L-arabinose metabolism. Such a system of linked operons has been termed a regulon (Maas and Clark, 1964).

The important characteristics of bacterial operons in terms of the control of gene expression can be summarized as follows:

(a) Cistrons coding for enzymes in the same pathways tend to be clustered and are often regulated co-ordinately by other genes. Such clustering is, however, a trend, not a general rule.

(b) Rate control over the expression of the operon may be effected by sensitivity of promoter genes to common cellular molecules such as cyclic-AMP.

(c) Operons work either positively or negatively or by some combination of the two.

Although not all bacterial enzymic pathways are known to be controlled as operons, it now seems likely that a large part of the prokaryotic genome is occupied by such gene groupings.

E. Sequential control of gene expression

This method of control depends on the transcription of some genes before others in the replicative cycle of the cell or virus and it is in studies of viral replication that the best examples are to be found. Of course, depending on the type of virus, single or double stranded, RNA or DNA, the replication of the viral genome itself proceeds in a variety of ways with a variety of intermediates. In most viruses studied, when nucleic acid production, either of an RNA or a DNA, is an early event in viral replication, the required polymerase is likely to be available from the virion itself (Baltimore, 1971). The existence of virion polymerases in some but not all viruses explains neatly why viral nucleic acid is sometimes infective (when the polymerase is not provided by the virion) and sometimes non-infective (when polymerase is provided by the virion). These various methods of viral nucleic acid replication do themselves imply some temporal control of transcription, in that nucleic acid replication may initially take precedence over protein synthesis. But the temporal control which is most evident in viral replication involves the selective transcription of the genome to produce early and late RNA and protein species. Actually, in some viruses there is no evidence for even this simple order of temporal control of genetic expression. This applies to single stranded DNA phages such as $\phi \times 174$ (Sedat and Sinsheimer, 1970).

A more representative example of viral multiplication is provided by the small coliphage T7. In this case there is excellent evidence that early transcription of part of the phage genome leads to the synthesis of a specific T7 RNA polymerase, the activity of which is essential for transcription of certain of the late genes (Chamberlain, 1970). It is not as yet certain whether this T7 polymerase turns on late genes by activating late promoter genes selectively or by reading through from the early genes. If the latter were correct, one would expect to find persistent synthesis of early RNA species. Summers and Sigel (1970) do not find this to be the case and conclude that transcription of the late genes is selective. T7 phage provides an example, generally applicable to the replication of most viruses, of temporal control of transcription. It is now appropriate to ask whether such a model is relevant to prokaryotic cellular organisms. To be more specific, is there evidence that, during the cell cycle, different enzymes are synthesized at different times? Of course such evidence would suffer from the objections that it might not represent temporal transcriptional control.

Analysis of temporal regulation of protein synthesis in bacteria has been undertaken chiefly through the use of artificially synchronized cultures of bacteria. Donachie and Masters (1969) have reviewed this approach very thoroughly. Like eukaryotic cells, growing bacteria have a cell cycle of G_1, S, G_2 and cell division, and the question posed is whether some enzymes are synthesized only or mainly during certain parts of the cycle and not others. The general conclusion which emerges from the evidence is that inducible enzymes may be induced with equal facility at all times and no period of the cell cycle is characterized by inhibition of the activity of particular genes. Of course, after S, the number of gene copies has doubled and, in step with this doubling, the enzyme levels also double, at least when equally induced, the step of enzyme doubling keeping time with the step of DNA doubling. However, certain enzymes appear to be regulated, not by external inducers but by internal timing mechanisms. Such enzymes are particularly those concerned with DNA replication and cell division (Helmstetter, 1969 and Lark, 1966).

It seems reasonable to conclude that although temporal control is important in the growth of many viruses, prokaryotic cells do not normally utilize this parameter in their genetic regulation. Instead, they rely on activation or repression of specific genes or groups of genes in response to the levels of other cytoplasmic molecules.

F. Multiple gene copies

Prokaryotic cells are conservative in the amount of their genetic material, and even some casual arithmetic would suggest that the haploid amount of DNA in *E. coli*, sufficient to code some 4000 different proteins as unique sequences, might possess, at most, a low level of multiple copies of single cistrons. Indeed, only in the case of genes coding for ribosomal and transfer RNAs is there any evidence for repeated gene sequences in bacteria. Mycoplasma, which is smaller and simpler than a bacterium, possesses only single copies of the ribosomal genes for 16S and 22S rRNA (Ryan and Morrowitz, 1969) but *E. coli* possesses five cistrons for the 16S and 23S rRNA, taking up some 0·2% of the genome (Kennell, 1965). As with the eukaryotic genome the ribosomal cistrons are linked and a polycistronic transcription unit combining the 16S and 23S genes is the primary product of the ribosomal genes in *E. coli* (Kossman *et al.*, 1971), as it is in eukaryotic cells. In both *E. coli* and *B. subtilis*, two separate regions of the genome can be identified with groups of 16S and 23S genes and cistrons for 4S (transfer RNA) and 5S (the small ribosomal RNA) are also present in these regions (Birnstiel *et al.*, 1971). However, the clustering may be fairly loose. In summary, although the genes for ribosomal and transfer RNA are repeated in as many prokaryotes as they are in eukaryotes, the repetition is rather small and there is no indication that it plays a major part in control rate of gene transcription.

An important aspect of the mechanisms of transcription in bacteria (and in many bacterial viruses) is the structure and function of the single form of bacterial RNA polymerase, but the interesting complexity of this enzyme will be discussed under the title of post-transcriptional control (page 24).

G. Recognition of base sequences by tertiary structure

Gierer made the interesting suggestion, in 1966, that an operator gene might contain some axial symmetry in its base sequence (palindromic sequences) permitting the formation of symmetrical loops. He further proposed that the repressor molecule might recognize the operator site, not by complementary pairing dependent on its base sequence, but by an interaction with the three dimensional structure formed by the loops. This suggestion is an extremely stimulating one for many aspects of nucleic acid function as a template, code and regulatory molecule; it seems possible that some structural genes might on occasion be recognized in this way and also that such a phenomenon might be involved in chromosome pairing at meiosis (Sobell, 1972).

Confirmation of the existence of axial symmetry in the nucleotide sequence of the lac operator has now come with the work of Gilbert and Maxam (1973) who find that a number of short sequences occur within the 24 base length of this piece of DNA which are axially symmetrical. Of perhaps greater interest is the finding of axially symmetrical regions at the ends and in the middle of the promoter region of the *E. coli* tyrosine transfer RNA gene, a region of 29 nucleotides in length (Sekiya and Khorana, 1974). These authors point out that, although no other *E. coli* promoters have been entirely sequenced, some have been partially determined e.g. those for the lac operon and for phage lambda. The other promoter regions mentioned are strikingly dissimilar to the tyrosine tRNA promoter in base sequence. This seems to imply that the RNA polymerase enzyme, which is presumably common to all these promoters and must, therefore, recognize each of them as a promoter, is unlikely to utilize base sequence for this function. It seems highly probable that the promoter in fact utilizes tertiary structure for promoter recognition and that all these promoters may be yet found to have similar tertiary structure though different base sequence.

H. Post transcriptional control

As has already been mentioned, transcription and translation are tightly linked in prokaryotes and the electron micrographs of Dr. Oscar Miller (see Fig. 2) demonstrate that many bacterial mRNA molecules commence translation before their transcription is completed. The lack of the nuclear envelope characteristic of the eukaryotic cell also prevents the nuclear processing and sieving of RNA molecules which is postulated in higher cells. However, certain post-transcriptional controls do affect gene expression in bacteria, although much of the current evidence is complex and somewhat confused. Two examples will be selected for discussion here in order to indicate the type of mechanism which operates at this level.

The first example is that of arginine biosynthesis in *E. coli.* This system has been discussed in depth by Vogel *et al.* (1971) and these authors have made a great contribution to understanding of this area. Synthesis of arginine in *E. coli* is mediated by eight enzymes and the synthesis of all eight is repressible by arginine in wild type strains of the bacterium. By the isolation of mutants with lesions in these enzymes, the structural genes have been mapped and, of the eight, four are clustered together and

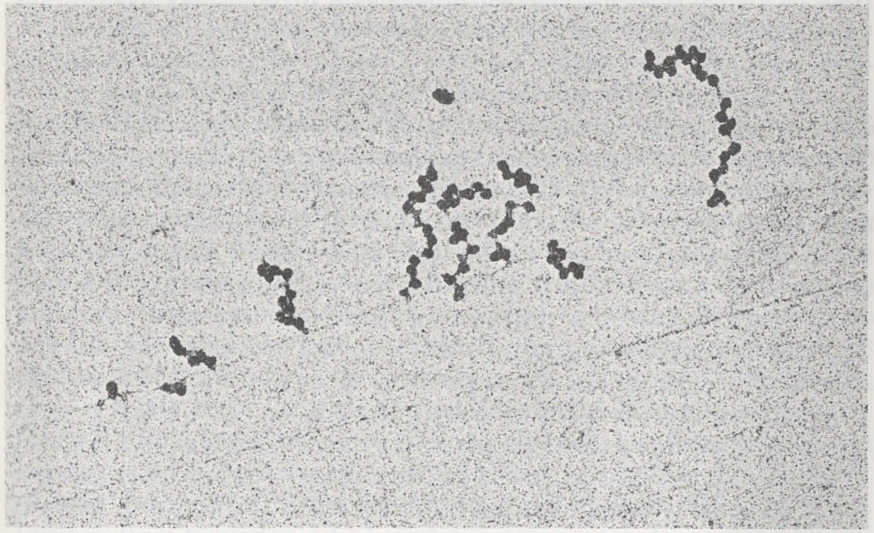


FIG. 2. Bacterial genes in action (from photographs kindly provided by Professor O. L. Miller; Miller, 1970; C 1970 by the American Association for the Advancement of Science). 2(a). An electron micrograph of a transcriptionally active gene of *E. coli*, showing ribosomes already attached to the mRNA molecules. Note that the mRNA molecules become longer as the polymerase enzymes traverse the length of the gene.

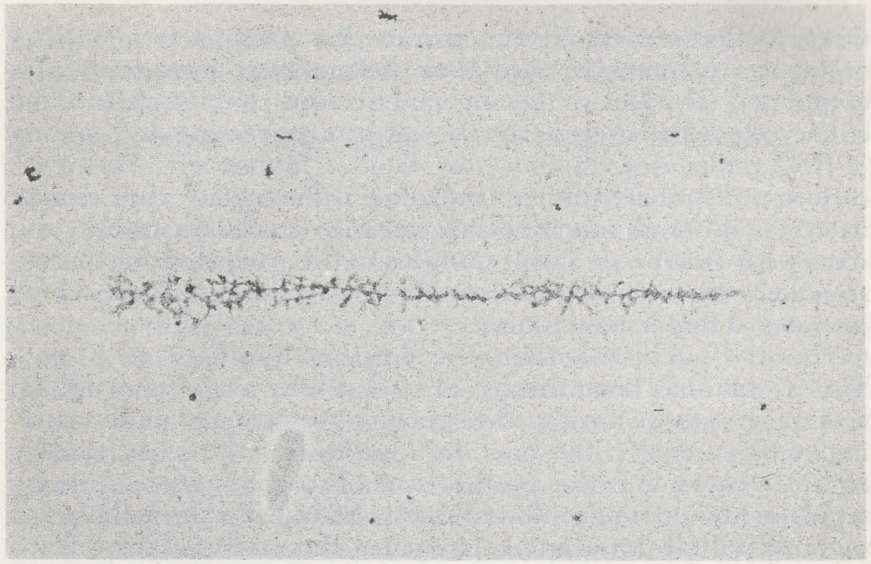


FIG. 2(b). An electron micrograph showing a portion of the *E. coli* genome presumed to be 16S and 23S ribosomal RNA loci. The fibrils attached to the DNA are therefore chiefly composed of lengths of ribosomal RNA, the lengths increasing as the polymerase traverses the gene.

four occur singly. In addition to these eight structural genes, there exists a system-specific regulatory gene R. Bacterial strains with a genetic defect in gene R are resistant to the arginine repression effect for all eight enzymes of the arginine pathways. Now it would be easy to conclude that this is simply another example of a centrally regulated series of positively switched operons, with arginine repressing the regulatory gene or inactivating the regulating gene product, and so depriving all the appropriate operons of the regulatory gene product. But certain items of evidence preclude such a conclusion. First, the R gene is found to specify a diffusible protein product (Jacoby and Gorini, 1969) which appears to be the arginine repressor and this repressor is only effective in the presence of arginine. Second, certain drugs such as streptomycin, known to be active at a ribosomal level, have specific effects on the arginine repression phenomenon (Vogel and Vogel, 1969). It now seems most probable that the explanation for a common effect on the products of the scattered genes of the arginine pathway is not to be found in a multiple operon model and transcriptional control (this was the earlier

interpretation of the evidence, Lewin, 1970), but in an effect at the level of translation, on the ribosome. The arginine repressor molecule, the messenger RNA for one of the arginine enzymes and probably other components, appear to interact and, in the presence of arginine, prevent translation of the messenger RNA molecule (Kryek and Rogers, 1972).

The second example of post-transcriptional control in bacteria which it is appropriate to discuss is that of assembly of the bacterial RNA polymerase molecule. The use of the term post-transcriptional is not absolutely correct here, since some aspects of the control are at, or near to, the transcriptional level. Highly purified bacterial RNA polymerase contains the following polypeptides β', β, σ, α and ω (Krakow *et al.*, 1969). The enzyme complex is readily dissociated into the core enzyme (lacking σ) and the sigma factor (σ), the intact complex being termed holoenzyme. The sigma factor is not essential for all enzyme activity, since the core enzyme will effectively transcribe calf thymus DNA (Burgess *et al.*, 1969) and the ω chain also seems to be dispensable. The core subunits B′, B and α are the only ones absolutely needed for RNA synthesis (Heil and Zillig, 1970). However, other factors are necessary for the specificity of transcription, particularly sigma. Just how sigma specifies transcription remains unclear, although it is presumed by most workers in the field to interact with the promoter gene complex. But the particular quality of sigma which is relevant here is that there is some evidence for different sigma factors having specificity for different promoters (Burgess *et al.*, 1969) although no great multiplicity of different sigmas has so far been revealed. Since sigma has the property of initiating polymerase core enzyme activity at one site and then detaching from that core in order to initiate elsewhere (Travers and Burgess, 1969a,b), the availability of sigma to core enzyme, occurring as it does after transcription, has an important role in determining gene expression. It also seems likely that the actual mechanism of action of sigma is to increase the efficiency of binding of the core enzyme to certain promoters, thus restricting their activity to certain sequences.

Another aspect of post transcriptional modification of this same enzyme is involved with the phenomenon of bacterial sporulation. Sporulation constitutes an important differentiated pathway for the bacterial cell and the synthesis of certain classes of messenger RNA is turned off and yet others turned on

(Yamagishi, 1968; Aronson, 1965). Such a switch in gene expression appears to be accomplished mainly by altering the specificity of the RNA polymerase enzyme, as suggested by Losick and Sonenshein (1969) and there is now evidence that the particular modification involves cleaving the β subunit of the polymerase by a special protease (Leighton ref. in Losick, 1972).

Many other post-transcriptional controls are known to operate in bacterial systems, some of them at a post-translational level. The controlled degradation of certain enzymes obviously constitutes an important mechanism in regulating what gene products are available, and in what quantity. All that has been attempted in this chapter is to indicate present understanding of a few selected bacterial systems and so provide an indication of how bacterial regulation is accomplished.

I. Expression of extrachromosomal genetic factors in bacteria

Bacteria harbour a variety of hereditary units which are physically separate from the main genome or chromosome of the cell. Since they play an important role in the life of bacteria and may be analogous to certain extrachromosomal factors in higher cells, it is necessary to examine their role in the genetic expression of the cell. Only relatively stable genetic units will be discussed and these include the sex factor (F), certain coliphages such as P1, colicin producing factors (normally signified by Col) and R factors which carry certain resistance determinants. Such extrachromosomal factors are now commonly termed *plasmids*, the term *episome* being reserved for plasmids proved to exist in a state of integration with the host cell chromosome as well as autonomously. A recent review of bacterial plasmids is that of Novick (1969) and Meynell (1972).

Bacterial plasmids have certain characters of particular relevance to gene expression. All consist of double stranded DNA and replicate out of synchrony with the S phase of the host cell. However, the degree of their replication is variable and the R factor in *Proteus* will account for 12% up to 33% of the total cellular DNA, depending on the state of growth of the cell (Novick, 1969). Bacterial plasmids may profoundly influence the characteristics of the host cell. For example, the Col factors produce colicins, proteins of variable complexity, which adsorb to specific membrane sites of some bacterial strains and induce the death of the affected cell. Thus, bacteria possessing Col factors become capable of inducing the death of certain other bacteria. A spectrum of plasmids can be drawn up from those which carry essential bacterial genes and are suggestive of 'escaped' parts of bacterial genome, to those which, like coliphage P1, are relatively intact bacteriophage. The position of sex factor F must be rather intermediate, but its important role in bacterial conjugation renders its influence on the host cell particularly crucial. So too with many of the R factors, extrachromosomal units conferring resistance to ultra violet light, phages, penicillin and a variety of other factors, apparently by coding for enzymes which decrease cell permeability or bind the particular toxins involved (Novick, 1966). Moreover, the plasmid is almost invariably dispensable to the cell in its normal functions (Novick, 1967) and so the expression of the plasmid genes endows the host cell with fortuitous new characteristics, some beneficial, some disadvantageous.

J. Comment

Bacterial genetic expression and its control, though undoubtedly simpler than that of eukaryotes, is highly complex and this chapter gives only an indication of the types of processes involved. Bacterial operons, the control of bacterial RNA polymerase function and plasmids no doubt have counterparts in higher cell systems, but there they are frequently obscured by other control mechanisms superimposed.

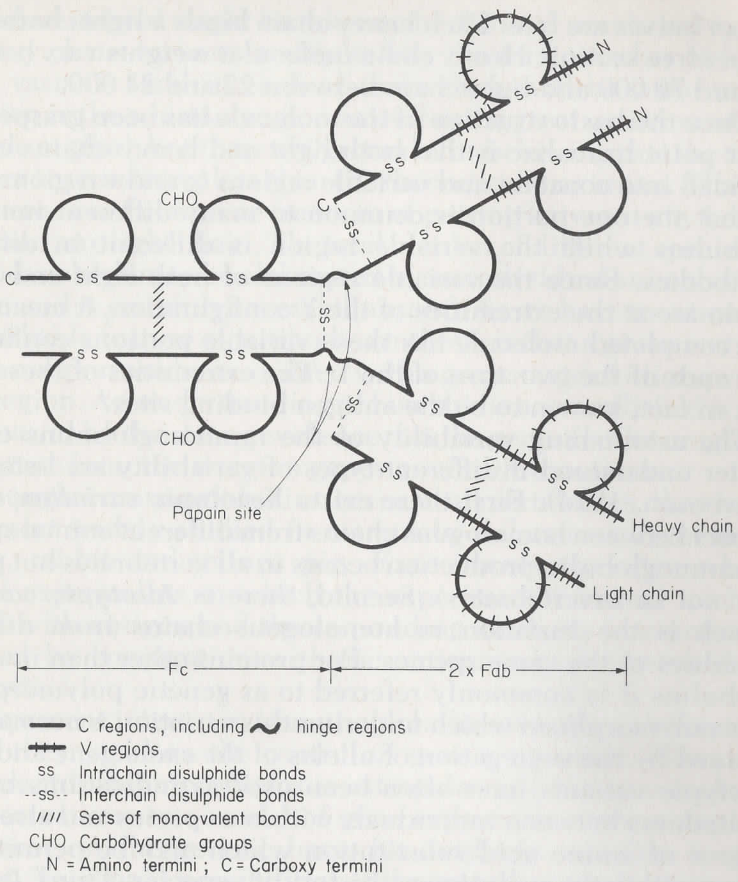
3. Experimental Systems of Differential Gene Function in Eukaryotes—Systems Involving One Type of Protein

A. Immunoglobulins 30
B. Lactate dehydrogenase 41
C. Haemoglobin 53
D. Lens crystallins 75
E. Milk proteins 83
F. Fibroin 88
G. Cocoonase 93
H. Vertebrate egg proteins 97

A. Immunoglobulins

The immunoglobulins provide a fascinating and useful research system to those interested in gene expression because of the enormous diversity of antibody type within a single animal and the large amount of information available at the molecular level. Unlike haemoglobins or cocoonase, which are attractive experimental systems because of their simplicity and the small number of gene products involved, the immunoglobulins are interesting and informative precisely because of the variation involved. In addition, antibody production boasts two phenomena, both pertinent to gene expression, which are probably unique to the immunoglobulins. These are first, allelic exclusion, the process by which only one of the two alleles of an immunoglobulin gene is normally expressed in any one lymphoid cell and, second, the association of separate genes, determining the variable and constant portion of the antibody molecule, in the synthesis of one polypeptide chain.

Antibodies are synthesized by cells of the lymphoid series, chiefly plasma cells. Some are exported by the cells and can be recovered free in the extracellular fluids of the body. Others remain attached to the surfaces of the antibody secreting cells. While the first group mediate the circulating or humoral antibody response, the second group are believed to be responsible for antigen recognition by the antibody-forming cell. Whether or not surface immunoglobulins are responsible for the phenomenon of "cell-mediated immunity" remains controversial.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Papain site
SS
Heavy chain
Light chain
Fc
2 x Fab
C regions, including ~ hinge regions
V regions
ss Intrachain disulphide bonds
-ss-- Interchain disulphide bonds
//// Sets of noncovalent bonds
CHO Carbohydrate groups
N = Amino termini ; C = Carboxy termini

FIG. 3. Diagrammatic representation of the four-chain structure of an immunoglobulin (antibody) molecule (from Stevenson, 1974).

A typical antibody molecule is now known to consist of a four chain molecule, as originally proposed by Porter (1962). An extensive review of antibody structure and present understanding of immunoglobulin production is to be found in Stevenson (1974). (See Fig. 3.) Two pairs of polypeptide chain are involved, one "light" chain pair and one "heavy" chain pair and within any single molecule the two light chains are identical and the two heavy chains are identical. The combined heavy chains take up the form of a Y, in which their lower halves are bonded and their upper halves are free. Each heavy chain binds a light chain on its upper free section. Heavy chain molecular weights vary between 50 and 70 000 and light chain between 22 and 24 000.

Once the basic structure of the molecule has been grasped, the next point to realize is that both light and heavy chains can be divided into constant and variable regions (c and v regions), that is that the one portion is common to many different immunoglobulins while the variable region is different in differing antibodies. Since the variable regions of both light and heavy chain are at the extremities of the Y configuration, it means that the completed molecule has these variable portions confined to the ends of the two arms of the Y. The extremities of these arms are, in fact, known to be the antigen binding sites.

The astonishing variability of the immunoglobulins can be better understood if different types of variability are listed (see Stevenson, 1974). First there exists *Xenotypic variation*, which occurs between homologous chains from different animal species (immunoglobulin production occurs in all vertebrates but probably not in invertebrates). Second, there is *Allotypic variation* which is the variation in homologous chains from different members of the same species. For proteins other than immunoglobulins it is commonly referred to as genetic polymorphism. The polymorphism which underlies this variation is normally explained by the segregation of alleles of the same gene and these allotypic variants have often been used as indications both of relatedness between individuals within a species and also of the degree of amino acid substitution which may be permitted to occur within the evolutionary history of a species. Third, *Isotypic variation* is the variation, within one individual of a species, in the sequences of the constant portions of the light and heavy immunoglobulin chains. Although these regions have been referred to as constant, there is in fact some variation detectable in the c regions in any one individual: this variation gives rise to the different immunoglobin classes IgG, IgM, IgA, IgD and IgE. As we shall discuss later, present evidence suggests that this variation arises from the fact that more than one gene copy exists for each piece of constant region. Last, and of the greatest significance in the overall variation of the antibodies, there is *Idiotypic variation*. This is variation within the variable regions of the chain and accounts for most of the incredible gamut of antibody species to be found in any one adult individual. The total potential variation in whole antibody molecules made by any one individual animal might be taken to be the total number of types of heavy chain variants multiplied by the total number of light chain variants (and in each of these there will be some small variation in the constant region as well as the great variation of the variable region), but there are grounds for believing that the total number is something less than the theoretical maximum. That is, that there is not complete freedom of pairing between any heavy chain and any light chain. The total number of different species of antibody which may be elaborated by an individual animal is not known and is certainly several thousand. As we have mentioned, a remarkable feature of antibody synthesis is that a given isotype for the c region can be associated with a great range of idiotypes of the v region. An understanding of how this is accomplished is not made easier by the strong evidence that the genes for the c regions are distant from the v region genes on the chromosomes.

Although immunoglobulins occur in all vertebrates, they have been most widely studied in mammals and especially in man, mouse, rabbit and guinea pig. Experiments are often whole animal experiments in which the experimental animal is challenged with antigen and the responding antibody is eventually detected by conjugation with the antigen which provoked its synthesis.

Our present interest in immunoglobulins is in the information which they yield on the control of gene expression. However, there are still major problems in extrapolating from immunological observations to a model of gene expression, since complete agreement on the genetic basis for antibody variation is still lacking. Two main possibilities still contend as mechanisms for antibody diversity. The first is termed the germ line theory and suggests that all the information necessary for the diversity is carried on genes inherited from the parents and that, therefore, an individual repertoire of immunoglobulins is the product of those genes of which activity and expression has been demanded by the antigens encountered. A development of the germ line theory is embodied in the clonal selection hypothesis (Burnet, 1969). This hypothesis states that an antibody forming cell, not only genetically equipped to make any of a whole range of antibody species but also predetermined or committed to the production of one particular species of antibody molecule, will, on receiving a stimulus from its complementary antigen, proceed to rapid division. Thus a clone of cells, all elaborating the same immunoglobulin, will develop rapidly in response to the presence of an antigen, with an equivalent rise in the titre of that circulating antibody.

A second main theory still commands attention, namely the somatic mutation theory. Both theories are discussed by Wigzell (1973) and Gally and Edelman (1972). This second theory suggests that, although some of the basis for antibody diversity would be inherited through the germ line, most would be generated during ontogeny by some form of somatic mutation. Such an elective procedure might, in some views, operate by a cell synthesizing an antibody whose sequence was only finally determined after scrutiny of the antigen. In other words that some modification of the genes for the variable regions could occur throughout life and often in direct response to and recognition of the appropriate antigen.

Whatever theory correctly accounts for the production of specific antibody in response to a particular antigen, there are two related questions about which much evidence now exists, although clear conclusions still evade us. These questions are: (1) how the c and v chromosomal regions, the one single and the other multiple, can come to be associated in the one polypeptide chain and (2) what genetic mechanism is responsible for v region diversity. Whether elected or selected, are v region genes as abundant as the different v regions and are they all grouped in one chromosomal area?

Stevenson (1974) has usefully assembled a list of eleven propositions on these questions, each proposition arising from a particular experiment or body of evidence. All that will be attempted here is a rather shorter form of Stevenson's catalogue. We will not attempt to review the evidence which underlies each statement. If it is not discussed, it can be assumed that the evidence is substantial and conclusive.

(a) The gene clusters for heavy and light chains are not linked and are both autosomal.
(b) Each different c region is the product of a separate cistron. In man and mouse the loci are clustered and in man some ten separate genes are involved.
(c) Different v regions appear to be the products of different genes, as judged by the evidence from sequence analysis.
(d) The different classes of light chains possess different sets of v regions, but different classes of heavy chains may share the same set of v regions.

(e) v region variations may be sorted out into sets and subsets, but even the subsets do not behave as alleles of the same gene.
(f) Hot spots of variability can be observed amongst the best studied v regions.
(g) Within a set or a subset of different v regions, it is found that, at least in rabbits, there is a sharing of allotypic characteristics which might be expected if they were under the control of a single locus. In other words, it is as if one particular allotype allele imposed itself on to the structure of a whole range of different idiotypes.
(h) There is strong evidence for linkage of the genes controlling v and c regions of either heavy or light chains. This statement compares interestingly with (a) which underlines the lack of linkage between light and heavy chain genes. Present evidence suggests that v–c gene crossovers for one chain can occur only as very rare events in either germinal or somatic transmission.
(i) The v regions of the heavy and light chains of any one antibody molecule are not related. That is, they are no more likely to be similar than the v regions of heavy and light chains of different molecules. Evidence for this proposition is, however, scanty.
(j) Allelic exclusion within single cells applies to allotypes of both the v region and c region. We should remark here that, apart from the inactivated X chromosome in many female mammals, allelic exclusion is not known to obtain regularly for the products of any genes besides those involved in immunoglobulin synthesis.
(k) Some evidence demonstrates that an immunoglobulin secreted by one cell clone may change its heavy chain c region and that cells synthesizing two distinct kinds of heavy chain c region may exist.

Now this catalogue of data provides a bewildering experience for anyone not well versed in immunoglobulin genetics and not all of it is immediately relevant to our topic. However, it does serve to underline the wealth of information which has been derived from this particular experimental system. Another point should also be stressed in our present context, namely that commitment to the production of one antibody species survives cell division. It is passed to all members of a clone of cells involved in elaborating antibody of one species. Without further discussion of the implication of all of these propositions, therefore, references will be made to them in the following discussion of topics particularly relevant to the control of gene expression.

1. Gene clustering

Light and heavy chain genes are separately clustered and not linked, while v and c regions for each are tightly linked. These observations imply that there may be largish blocks of chromatin on at least two chromosomes devoted to immunoglobulin synthesis. In fact present evidence suggests that three blocks on three chromosomes are involved: one block for heavy chains, one for light chains of class κ and one for light chains of class λ. Just how large these blocks might be depends largely on our estimation of the extent of the v region variability. If all v regions are indeed the product of separate cistrons and each cell makes at most two (one for the light and one for the heavy chain), then this is an astonishing example of really impressive gene control—one of a whole series of related genes being turned on while all its neighbours are off. The clustering can be assumed to result from gene duplication and mutation in the course of evolution, together with some constriction on the scatter of the genes, imposed by the necessity for v and c genes for any one chain to co-operate in transcription.

2. Allelic exclusion

Aside from X chromosome inactivation and tRNA synthesis in hybrid *Xenopus* (see Section 1A), no other case of allelic exclusion is known, nor is it easy to envisage a mechanism to account for it. Let us recall what is involved. The homologous chromosomes are distinct and perhaps distant from one another in the interphase chromatin, yet, within any single cell, only one of the two alleles of the immunoglobulin gene are active. Moreover, this unilateral expression is handed on to the progeny of a cell so committed. The problem is perhaps less serious for a selective theory of antibody synthesis since it would be conceivable that no opposite number existed for the gene, that is that a novel sequence was somehow established on one chromosome only, in response to recognition of one antigen.

On the other hand, even with a selective theory, if only one molecule of antigen were permitted to enter the lymphoid cell, that single molecule might act as an inducer in such a limited way that only one of the two genes present (of which the polypeptide product would match the antigen molecule) could be activated. Although such limited induction is difficult to understand there is some evidence for such an activity in the genetics of phase variation in *Salmonella* (discussed by Cook, 1974). In this example only one of two allelic forms of a gene is active at one time in the bacterium, the differential activity apparently being affected by the position of the genes and by the activity of other genes on the same chromosome. The *Salmonella* example does not necessarily depend on shortage of an inducer molecule at the site of the gene concerned, but on the activity of genes elsewhere on the same chromosome.

It is important to recognize that not too much weight should be given to the earlier statement that allelic exclusion was unique to immunoglobulin synthesis. For the fact is that it has rarely been sought with other proteins and the immunoglobulins provide a system in which it can be very readily detected. Nevertheless, many other eukaryotic genes clearly do not operate in this way. For example, persons heterozygous for the sickle form of the Beta chain of adult haemoglobin would, with allelic exclusion, have some blood cells which sickled easily (where the S allele was active) and some which sickled not at all (where the normal Beta allele was active). This is not the case. Persons heterozygous for this gene possess blood cells all of which will sickle slightly on exposure to low oxygen tension (see discussion in Maclean and Jurd, 1972).

3. Preservation of allotypic identity with idiotypic variation

One of the most intriguing and perplexing observations in immunoglobulin genetics is that mentioned in proposition (g) above. Such a phenomenon is curiously reminiscent of the master-slave models of eukaryotic genetic organization suggested by Callan (1967). See the discussion in Maclean, 1975 (The Differentiation of Cells). In the present example, the master gene would correct the copies only in so far as certain amino acid sequences were concerned, namely the ones involved in determining the allotypic identity. Unfortunately there is now a substantial body of evidence which argues against master-slave models as a regular feature of the organization of chromosomes. Nevertheless, mechanisms do apparently exist for preserving the identity of long series of gene copies against the effects of evolutionary divergence. This is best seen in the case of the many copies of ribosomal and transfer RNA genes in *Xenopus*. As remarked by Birnstiel *et al.* (1968), the many copies of these genes in this organism are extremely similar, apparently in defiance of random mutation. The matching procedure would involve either a circularization of the chromosome to permit copying of the master sequence, or a transferable piece of genetic information, either RNA in nature with a reverse transcriptase enzyme active, or DNA in nature with a DNA polymerase. A mechanism to account for the complementation of the separate v and c regions has been suggested by Gally and Edelman (1972), being a form of somatic translocation. This will be discussed more fully in a following section. Gene matching, partial or complete, is, therefore, a process suggested by genetic evidence from a number of different sources.

4. Somatic mutation

Some form of somatic mutation has been suggested by many authors as an explanation for idiotypic variation in antibody structure. It has the advantage of reducing the need for vast numbers of distinct immunoglobulin gene sequences and would, in at least some schemes, permit antibodies to be custom built to antigen specification by tailoring appropriate gene sequences. Unfortunately, evidence in favour of a somatic mutation process is hard to find. On the other hand, there is some evidence that cells which fit an antigen, at least in terms of cell surface molecules, exist prior to the addition of the appropriate antigen (Davie and Paul, 1973). Although one search for somatic mutation within a clone of lymphocytes has drawn a blank (Askonas *et al.*, 1970), a recent report of apparent somatic mutation within clones of immunologically committed cells has appeared (Cunningham and Fordham, 1974). What certainly cannot be excluded at the present time is a process of somatic mutation which would act prior to cell exposure to antigen. Its purpose, therefore, would not be to tailor a gene and its product to the configuration demanded by an antigen, but simply to increase immunoglobulin gene diversity in lymphocytes, perhaps along with specific gene duplication. This would avoid the need for a large number of antibody gene sequences to be transmitted in the germ plasm. Recent hybridization studies (Leder *et al.*, 1974) certainly argue against a strict germ line theory as an adequate explanation of antibody diversity.

5. Translocation

One particular form of somatic mutation requires closer examination, namely translocation. This mechanism has been invoked by immunogeneticists not so much to explain the richness of antibody diversity but to account for the complementary synthesis of one polypeptide chain by v and c region genes which are distant from one another on the chromosome. The problem and its possible solution by this model have been discussed by Gally and Edelman (1972). These authors have proposed a fundamental genetic element which they term a "translocon". Such a unit is visualized as a chromosomal segment which contains all the structural information for one particular "type" of immunoglobulin, which sequence would be transmitted from one generation to the next. A translocon would, therefore, be a gene cluster of immunoglobulin information cistrons, as discussed in the section on gene clustering, and would possess genes for both c and v regions. When a translocon becomes active in terms of immunoglobulin synthesis, a somatic translocation is visualized which would bring into juxtaposition a v region gene and a c region gene. Gally and Edelman (1972) propose that the translocation would be effected by the excision of a v region gene and its later insertion next to a c region gene, and they list a number of lines of evidence favouring such a model. One of the most persuasive of these is the switchover event. Not uncommonly, and when an animal is initially challenged with antigen, IgM (Immune gamma globulin M) antibody activity can be detected prior to the appearance of IgG antibody. Studies have shown that individual lymphocytes may contain both and are apparently caught while switching from one to the other (Nossal *et al.*, 1964). These sequential IgM and IgG antibodies from a single clone are believed to have identical light chains, identical heavy chain v regions, but different heavy chain c regions. It is extremely difficult to see how precisely the same v region DNA sequence can be consecutively combined with two different c region sequences except by some sort of translocation, or at least by some contrived manipulation of the sequences which would bring them into linear juxtaposition. For the purposes of this book one of the more exciting aspects of the translocon hypothesis is that some other biological systems also seem to demand such a model for their operation. First, it is known that viruses and phage may have their genomes inserted into the genomes of eukaryotes or bacteria so that they come to be read in sequence with the host genome. They may even be inserted within a gene and transcribed along with that gene. Second and perhaps more cogently, somatic rearrangement of chromosomal material has been postulated to occur in some other examples, e.g. in maize (McClintock, 1965), *Drosophila* (Green, 1969) and in *Salmonella* (Smith-Keary and Dawson, 1964). The bacterial evidence suggests an episome like element which does not affect the expression of the gene with which it associates, but which affects its mutability (it seems possible that such a model might also have something to give to the immunoglobulin situation). Similarly, in *Drosophila*, the mutation of the w gene is affected, but in addition, examples were found where the episome had apparently been inserted elsewhere in the genome and carried part of the w region with it. The closest parallel in evidence and ideas exists between immunoglobulin translocons and control element genes in maize. The maize evidence suggests highly mobile genetic elements which may be inserted in a number of different chromosomal locations, and which also may be excised and reinserted elsewhere within the life and cell division of the somatic tissues. Not only would these elements profoundly affect the mutation rate of the neighbouring genes, but also directly affect their expression in transcription.

6. Cellular control of antibody production

We will discuss only briefly another aspect of immunoglobulin production which has a bearing on the control of gene expression, namely the communication between different cell types and cellular responses to signal molecules, both of which constitute the responsiveness of the immune cell system. It is now known that there are two distinct classes of lymophocytes, the so called T cells which undergo mitotic expansion in the thymus (Roitt *et al.*, 1969) and the B cells which are the antibody forming cells and are produced from non-thymus sources such as bone marrow. The co-operation of the T and B cells in mediating antibody production in response to antigen has been widely discussed, e.g. Gershon (1974). Present evidence suggests that the T cell does not make an antibody but is itself sensitive to circulating antigen, both qualitatively and quantitatively. The nature of the binding of antigen to the T cell determines the instructions which the T cell gives to the B cells, which actually make the antibody. Moreover, the sensitivity of the T cell is not only to circulating antigen but also to signals from macrophages, basophils and probably other T cells and feedback signals, such as antibody–antigen complexes, which may lead the T cell to turn off the antibody response.

The T and B lymphocyte system, therefore, includes a complicated system of inter-cellular signalling, some mediated by surface attaching molecules, others perhaps by cell to cell interactions involving surface contact. These signals serve to influence the expression of the cellular gene endowment, either transcriptionally, post transcriptionally or both. The extent to which the immune system is a model for cellular gene expression in general is uncertain, but as a system for the investigation of cellular signalling in gene expression it is surely unrivalled.

7. Comment

Studies on immunoglobulins provide us with a wealth of genetic information and it will be surprising indeed if an understanding of such phenomena as allelic exclusion and v and c region complementarity do not throw a lot of light on other genetic systems. The immutability of the genome in somatic tissues is too often taken for granted.

B. Lactate dehydrogenase

This protein is an enzyme which exists in a number of different forms, separable by electrophoresis and other techniques, but all doing essentially the same metabolic job. They are, then, isoenzymes (sometimes also termed isozymes). Lactate dehydrogenase (LDH) consists of four subunits, more than one cistron being involved in coding for these subunits. Moreover, different tissues in the same organism possess varying proportions of the LDH isoenzymes, as also does the same tissue during different stages of ontogeny. It is, therefore, easy to see that a study of LDH in different tissues and at different developmental stages should provide interesting information about gene expression in these situations.

Most tissues of most mammals yield five easily separable LDH bands in agar or polyacrylamide gel electrophoresis and these separate isoenzyme bands are normally styled bands 1, 2, 3, 4, 5. Each is a tetramer of four polypeptide chains. In any given species all of the isoenzyme LDH tetramers have the same molecular weight—about 135 000, as measured by centrifugation and light scattering.

Drastic hydrolysis of LDH isoeyzmes, followed by amino acid analysis, betrays differences in the amino acid composition of different bands in the same organism (Wieland and Pfleiderer, 1961). If the bands are treated more gently with 12M urea, the tetramers break and the monomers run singly in the electrophoretic analysis. This demonstrates that all five LDH isoenzymes possess only two different monomer subunits, termed A and B (Markert and Appella, 1963). The two subunits give rise to the five tetramers by their presence in varying proportions, so that LDH (1) is B_4 (i.e. four B subunits in a tetrameric configuration), LDH (2) is B_3A_1, LDH (3) is B_2A_2, LDH (4) is B_1A_3 and LDH (5) is A_4. If a mixture of subunits is made from purified LDH_1 and LDH_5, and the monomers permitted to associate in molar sodium chloride, then all five isoenzymes can be detected in the solution in the proportion of 1 : 4 : 6 : 4 : 1. These figures are indicative of random association (Markert, 1963). There is also evidence that monomers and dimers are not enzymatically active, only the complete tetramer.

As remarked above, although monomers will give a 1 : 4 : 6 : 4 : 1 ratio of the isoenzymes if allowed to associate freely from equal amounts of the subunits, analysis of LDH in most mammalian tissues shows widely differing ratios, indicating that in some tissues there is an excess of A over B subunit and in other tissues vice versa. It is, therefore, clear that a study of LDH isoenzyme patterns might provide us with information about the finer modulation of gene expression—the rate control over production of the two separate gene products.

Before reviewing the evidence about variable synthesis of the A and B subunits in different tissues and at different times, it is also relevant to mention that certain other LDH isoenzymes have been found besides the basic five already mentioned. One styled LDH_x, found in testis of man and other animals, appears to be related to spermatogenesis. The structure of LDH_x, which is, like other LDH molecules, a functional tetramer, appears to be that of four identical X sub-units, now more frequently termed C subunits (Wilkinson and Withycombe, 1965). An interesting complication to LDH_x has been found in the pigeon. In this bird a mutant form of the C sub-unit exists as an allele to the normal formal, some birds being homozygous and others heterozygous for the alleles. Thus pigeons homozygous for the normal wild type C gene show one LDH C_4 tetramer band on electrophoresis, pigeons homozygous for the mutant C gene allele have a single LDH C_4^1 tetramer which is electrophoretically distinct from normal C_4 isoenzyme and the heterozygous birds show all five bands of C_4^1, $C_1C_3^1$, $C_2C_2^1$, $C_3C_1^1$ and C_4 in an expected distribution of 1 : 4 : 6 : 4 : 1, indicative of a random aggregation of the two types of sub-unit (Zinkham *et al.*, 1966).

The isoenzymes of subunits A, B and C are fairly generally distributed amongst birds and mammals, but another type of subunit, termed E, occurs in many species of fish, confined apparently to the cells of the retina and certain nerve cells of the brain. Like the C gene of mammals and bird spermatocytes, the E gene of fish is apparently active only at certain developmental stages and only in a few cells, since the presence of the LDH-$_E$ isoenzyme can only be detected in adult fish. The E tetramer consists of four identical E monomers. In addition, the E monomers will hybridize with A and B monomers to yield other mixed LDH isoenzyme bands. Also of interest is the observation that in some species of fish the normal A and B subunits do not co-polymerize, hence only LDH_1 and LDH_5 normally occur in their tissues. However, in their brain and retinal cells, where subunit E is also synthesized, a range of isoenzymes can be detected because of the co-polymers of E with A and E with B (Markert and Ursprung, 1971).

The activity of the genes coding for the LDH subunits presents two separate problems, assuming, that is, that the differential occurrence of the subunits is a true reflection of gene activity and is not complicated by other processes of post-transcriptional control. This point we will return to later in this section. The two problems are, first, the apparent restriction of the activity of the genes for the C and E subunits to particular tissues and particular developmental periods and second, the unequal expression of the A and B subunit genes in different tissues and at different times.

These two cistrons appear to be active in almost all tissues at all times but their rate of activity varies. These two problem areas will be discussed separately.

1. LDH subunits with very restricted expression

As mentioned above, the subunits C and E are restricted in their appearance, the first appearing only in the spermatocytes of birds and mammals and, therefore, synthesized for a brief ontogenic period, the second only in certain eye and brain cells of adult fish. To take LDH_C first, not only is it exclusively present in the spermatocyte (and therefore, of course, in the adult testis) but the normal A and B subunits are not expressed in the spermatocyte. In other words, the avian and mammalian spermatocyte apparently has unique LDH requirements. It is known that the LDH_C form has broader substrate specificities and kinetic properties than have the other five isoenzymes (Wilkinson, 1970). Evidence from work on the pigeon also seems to emphasize for how short a time the LDH_C synthesis is active. If pigeons which are heterozygous for the mutant C subunit gene are assayed by homogenization of the testis, then a random 1 : 4 : 6 : 4 : 1 occurrence of the LDH C bands is found. This picture persists even if all necessary precautions are taken to ensure that the groupings which are assayed are those which occurred within the cells. Now since these birds are heterozygous, the reduction division of meiosis involved in sperm production will ensure that all sperm carry only the C or C^1 gene and would, therefore, only be capable of elaborating the homopolymer, C_4^1 in some sperm and C_4 in others. The random distribution of the five bands of LDH_C must imply that synthesis of the monomers ceases before the reduction division. This implies a very short synthetic period, perhaps only a few hours. Here then is a gene only ever expressed for a few hours in the life of an organism and that prior to the union of sperm and egg. Moreover, for that period the genes for A and B are not expressed, the production of the A and B subunits ceasing in the spermatogonia.

In many ways the pattern of LDH synthesis and occurrence is very similar to the haemoglobins, in that both are tetrameric molecules made up of four subunits and with ontogenic changes in expression during embryonic, foetal and adult life. LDH does, however, provide one facet additional to the haemoglobin picture, following from its much more ubiquitous distribution in the body tissues. The additional facet is the alteration in the location of synthesis, since the LDH E subunit is confined to brain and eye tissue of adult fish and LDH C to primary spermatogonia of birds and mammals. These examples serve to impress us with the knowledge that genes may occur in the genome which are silent for almost the whole of ontogeny in all cells. How many genes, one wonders, are present in the genome and are never ever expressed in the life of the organism. Evolution might eliminate the expression of a useless or troublesome protein by ensuring constant repression of that gene rather than by eliminating it from the genome.

However, one other curious aspect of LDH biology also mirrors the haemoglobins, namely that when, in early development, a distinct molecular species makes its appearance, e.g. LDH C or embryonic or foetal haemoglobin, the other adult forms of these molecules are not made. Expression of one gene is paralleled by lack of expression of the others. It would be fascinating to know whether the two events are linked, or whether the system is leaky, so that some spermatocytes do make LDH A and B subunits or some human embryos make Beta globin chains. We will return to discuss this question further in the section on haemoglobins.

2. The distribution of the A and B type LDH subunits

If LDH is extracted from tissue homogenates by a gentle procedure, the tetrameric form which occurred within the cell is retained. The composition of these tetramers may then be determined by electrophoresis, since the position of the five alternative tetrameric arrangements may be ascertained from control runs. Two distinct phenomena are evident from a comparative study of these five isoenzymes. The first is that, in the adult animal, different tissues display widely differing patterns and the second is that any one tissue may vary its pattern during ontogeny. We will discuss the phenomena separately in the order mentioned, although clearly, in nature, the two processes belong together as part of an overall modulation of the production of the A and B subunits.

It is well to ask at the outset of such a discussion whether, within a cell or tissue, the subunits aggregate randomly. The short answer is that, in most cases, they do, but sometimes they do not. For example the LDH pattern from rat kidney reveals a predominance of the LDH 1 and LDH 5 bands and only a small yield of the 2, 3 and 4 isoenzymes. Such distributions may result either from non random subunit polymerization due to the prevailing cytoplasmic environment or to variable degradation rates of some of the isoenzymes. Such points will be discussed later. For the present, the tissue patterns which do conform to random subunit polymerization will be compared.

3. Different tissues of the adult animal display different LDH patterns

Table 1 and Fig. 4 demonstrate very clearly the very wide range of LDH variation obtaining in different tissues of one organism. Thus, the heart tissue has predominantly subunit B in evidence and skeletal muscle subunit A. There is fairly general parallelism for the patterns prevailing in one tissue of different animal species, comparing, for example, heart muscle in man, rat, rabbit and monkey, although subunit A is not evidently expressed in rabbit heart, but is to a limited extent in human heart. On the other hand the heart tissue of chickens is almost exclusively synthesizing subunit A. Several laboratories have investigated the absence of LDH_5 from red blood cells and it now seems clear that these cells lose their complement of A subunit while retaining the B (Starkweather *et al.*, 1965). Since the synthesis of LDH ceases in the mature erythrocyte, the early degradation of subunit A leaves a population exclusively made up of subunit B which goes to form the tetramer of LDH_1. The favoured explanation for the predominance of LDH_5 or LDH_1 is that they have slightly different properties, the type A subunit being geared to the reduction of pyruvate and the type B subunit to the oxidation of lactate (Everse and Kaplan, 1973). Therefore, tissues involved mainly in pyruvate reduction would have type A subunits, i.e. mainly LDH_5, while tissues involved mainly in the oxidation of lactate would prove to be rich in type B subunits, i.e. mainly LDH_1.

This theoretical basis of the tissue distribution of the A and B subunits does seem to square with most of the evidence. Thus, skeletal muscle is rich in LDH_5, the type A subunit, while heart muscle is rich in LDH_1, the type B subunit. It is also interesting to note that some species with particularly specialized life styles may lack one of the subunits entirely. Both the lamprey and the halibut lack the type B subunit in all tissues, a fact probably

TABLE 1. Distribution of lactate dehydrogenase isoenzymes in various species and tissues (from Wilkinson, 1970). Figures have been calculated from results obtained by many different authors.

		% distribution of LD isoenzymes					
Species	Tissue	LD_1	LD_2	LD_3	LD_4	LD_5	References
Human	Heart	35	36	12	16	11	[1, 2]
		67	29	4	<1	<1	[3]
		71	27	2	—	—	[4]
		49	45	6	—	—	[5]
		53	29	16	2	<1	[6]
	Kidney	12	14	24	25	23	[1, 2]
		42	48	9	1	—	[4]
		30	50	15	5	—	[5]
		52	28	16	4	<1	[7]
	Erythrocytes	39	56	—	5	—	[1, 2]
		39	46	15	—	—	[3]
		46	39	11	4	—	[4]
		42	36	15	5	2	[7]
		36	44	14	4	2	[14]
	Brain	21	26	26	20	8	[2, 8]
		25	25	34	15	1	[9]
	Adrenal	3	20	75	—	2	[5]
	Lung	10	20	30	25	15	[5]
	Lymph node	10	25	60	—	5	[5]
	Pancreas	30	15	50	—	5	[5]
	Placenta	12	18	15	30	25	[1, 2]
	Thymus	10	11	30	28	21	[1, 2]
	Thyroid	12	25	55	—	8	[5]
	Spleen	6	11	35	28	20	[3]
		10	25	40	20	5	[5]
	Leucocytes	11	58	27	4	—	[4]
		8	12	50	18	12	[7]
		6	19	28	19	22	[15]
	Skeletal muscle	4	7	21	27	41	[1, 2]
		—	—	2	1	97	[3]
		—	—	17	10	73	[4]
		16	18	18	12	36	[5]
		<1	15	38	36	11	[6]
		1	4	8	9	78	[10]
	Liver	2	4	11	27	56	[1, 2]
		—	6	12	6	76	[3]
		8	10	33	13	36	[4]
		2	5	12	—	81	[5]
		<1	10	19	17	54	[6]
		2	2	3	12	80	[10]
	Uterus	5	25	44	22	4	[13]
	Uterus (gravid)	2	5	28	45	20	[13]

continued

TABLE 1—*continued*

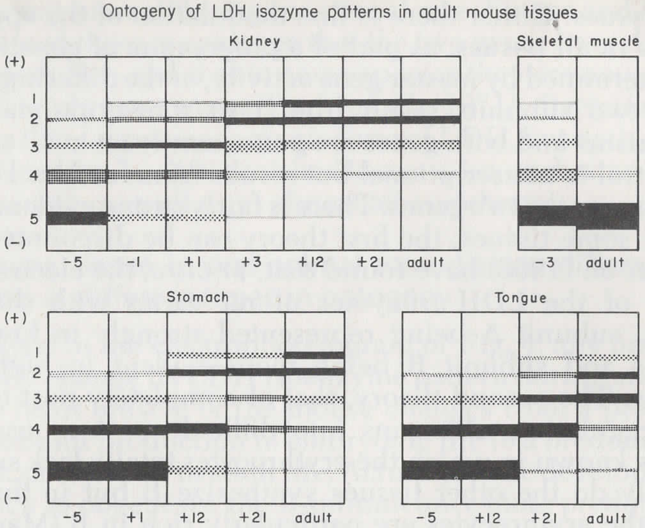
Species	Tissue	% distribution of LD isoenzymes LD$_1$	LD$_2$	LD$_3$	LD$_4$	LD$_5$	References
Rat	Heart	50	30	12	6	2	[10, 11]
	Brain	40	20	15	20	5	[10, 11]
		39	20	18	18	5	[12]
	Kidney	20	15	10	20	35	[10, 11]
	Skeletal muscle	1	1	3	5	90	[10, 11]
	Liver	1	1	3	5–10	85–90	[10, 11]
Rabbit	Heart	100	—	—	—	—	[3]
		94	2	1	3	<1	[10]
	Erythrocytes	100	—	—	—	—	[3]
	Kidney	77	11	7	3	2	[3]
		63	10	8	9	9	[10]
	Spleen	43	43	14	<1	<1	[3]
	Brain	43	20	27	6	4	[3]
	Lung	23	35	21	4	17	[3]
	Liver	2	4	24	59	11	[3]
		1	3	17	39	40	[10]
	Skeletal muscle	—	—	—	—	100	[3]
		<1	<1	<1	3	95	[10]
Monkey	Heart	35	20	13	15	16	[2]
	Erythrocytes	29	49	15	7	—	[2]
	Lung	18	24	18	17	23	[2]
	Spleen	13	15	15	23	34	[2]
	Liver	7	8	13	28	43	[2]

The above figures have been calculated from results published by the following authors:

- [1] Wieme (1959)
- [2] Wieme and Van Maercke (1961)
- [3] Plagemann *et al.* (1960*a*)
- [4] Vesell and Bearn (1961)
- [5] Wróblewski and Gregory (1961)
- [6] Plummer *et al.* (1963)
- [7] Withycombe (1965)
- [8] Löwenthal *et al.* (1961)
- [9] Gerhardt *et al.* (1963)
- [10] Wieland *et al.* (1959)
- [11] Wieland and Pfleiderer (1961)
- [12] Bonavita *et al.* (1962)
- [13] Geyer (1968)
- [14] Starkweather *et al.* (1965)
- [15] Malasková and Holeyšovská (1969)

related to their low metabolic rate and dependence on glycolysis (Wilson *et al.*, 1964).

Clearly, there are three main theories which could be advanced to account for the varying distribution of the subunits in different


Ontogeny of LDH isozyme patterns in adult mouse tissues
Kidney
Skeletal muscle
(+)
1
2
3
4
5
(−)
−5
−1
+1
+3
+12
+21
adult
−3
adult
Stomach
Tongue
−5
+1
+12
+21
adult
−5
+12
+21
adult

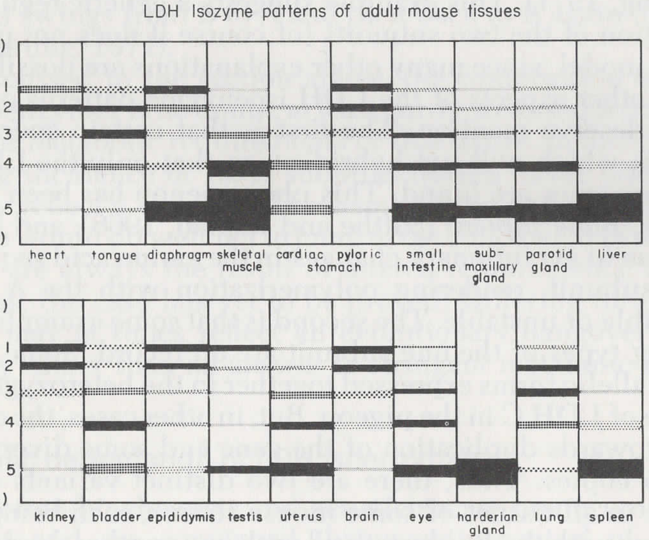

LDH Isozyme patterns of adult mouse tissues
(+)
1
2
3
4
5
(−)
heart
tongue
diaphragm
skeletal muscle
cardiac stomach
pyloric stomach
small intestine
sub-maxillary gland
parotid gland
liver
kidney
bladder
epididymis
testis
uterus
brain
eye
harderian gland
lung
spleen

FIG. 4. Ontogeny of LDH isozyme patterns in mouse tissues. The figure is a diagrammatic representation of the changing LDH pattern during the development of several mouse tissues. Note that the patterns do not change synchronously. The numbers along the abscissa indicate days before (−) or after (+) birth.

4(b). LDH isozyme patterns of different adult mouse tissues. The figure is a diagrammatic representation of the LDH zymogram pattern from twenty tissues or organs of the adult mouse. The intensity of the isozyme band is an indication of the amount of enzyme activity in the band.

Both Figures from plates kindly lent by Professor C. Markert (Markert, 1967).

adult tissues. Either there is fine modulation of the appropriate cistrons in all tissues, as part of a programme of genetic expression determined by master gene activity, or the differing amounts of the two subunits result from post transcriptional control mechanisms and both genes are permanently on in all tissues, or the control is transcriptional but results from feed back from the cytoplasm to the two genes. There is fairly strong evidence that, at least in some tissues, the first theory can be discounted. Goodfriend *et al.* (1966) have found that, *in vitro*, the electrophoretic pattern of the LDH isozymes of rat varies with the oxygen tension, subunit A being represented strongly in low oxygen tensions and subunit B being more evident in high oxygen tensions. The second theory, that of completely post transcriptional regulation, also runs into difficulties. A mouse mutant strain is known in which the erythrocytes totally lack subunit B. Not only do the other tissues synthesize B but in the normal mouse the erythrocytes are particularly rich in B (Markert and Ursprung, 1971). This example suggests a genetic regulation of production of the two subunits (of course it does not prove the genetic model, since many other explanations are possible).

Two other aspects of the LDH isoenzyme patterns of animal tissues deserve mention. The first is that certain species have subunits which will not hybridize, so that only the LDH_1 and LDH_5 enzymes are found. This phenomenon has been noted in the frog, *Rana pipiens* (Salthe and Kaplan, 1966), and is apparently due to evolutionary changes in the amino acid sequence of the B subunit, rendering polymerization with the A subunit impossible or unstable. The second is that some examples of two differing types of the one subunit are on record. Some of these may be allelic forms expressed together in the heterozygote, as in the case of LDH C in the pigeon. But, in other cases, the evidence points towards duplication of the gene and some divergence of the two copies. Thus, there are two distinct variants of the A subunit in all tissues of some mouse strains, each being able to hybridize with the B subunit and with one another, leading to no less than fifteen different LDH isozyme bands in these mice (Costello and Kaplan, 1963). Two A subunits occur in the haddock which hybridize with one another but not with the B band, yielding a total of six isoenzyme bands, five of which comprise A subunits only (Kaplan, 1968).

It is, therefore, apparent that in different adult tissues, there are differing requirements for the two gene products and that each

LDH subunit has a differing functional rate (Everse and Kaplan, 1973). Indeed there is evidence that the two genes have diverged early in evolution and that in some species they are now located on separate chromosomes (Puck *et al.*, 1971). Bacteria, slime moulds and fungi possess a single form of LDH, but both A and B subunit types can be distinguished in many invertebrates, e.g. the horseshoe crab, as well as in almost all vertebrates.

4. The same tissue in one animal may display different LDH patterns at different times in ontogeny

As displayed in the electrophoretogram of Fig. 4, any one tissue may greatly change its LDH isoenzyme pattern during ontogeny. Thus the heart muscle of the mouse changes from a predominantly A subunit production in embryonic life to a predominantly B subunit production in adult life. Actually, if the development is traced back to oogenesis, the B subunit once more predominates so that, during heart muscle differentiation in the mouse, the synthesis swings from B to A and then back to B again (Markert and Ursprung, 1971).

As discussed in the section above, the composition of tissue LDH isoenzymes is no doubt, at least partly, a consequence of the particular metabolic requirements of that tissue. In this light the changing incidence of LDH subunits during tissue differentiation may reflect a changing biochemical demand. On the other hand, we would do well not to jump to the conclusion that altered patterns are always the result of altered requirements. In most situations, the case has yet to be proved. Differing forms of an enzyme may at times reflect an evolutionary hangover during ontogeny rather than an answer to stringent metabolic requirements.

5. Specific degradation of LDH isoenzymes

Far too often biochemists and geneticists are preoccupied with synthesis and are convinced that genetic control of protein synthesis chiefly governs the life of a cell or an organism. This is almost certainly a very blinkered view of cellular dynamic biochemistry, as stressed in recent excellent reviews by Schimke (1973) and Goldberg and Dice (1974). Schimke quotes half lives of common enzymes in cells which vary from 11 mins for ornithine decarboxylase to 16 days for LDH_5, with many other enzymes falling between these two extremes. It is also clear that their variant half lives are not simply a function of differing inherent protein stabilities, but that controlled degradation of one enzyme by another is a highly important aspect of cellular metabolism. In the widest sense of that phrase, it is also a very important aspect of genetic expression and its control. Studies with LDH have proved particularly useful in determining the significance of controlled degradation; a number of distinct facts emerge. The first is that the rate of degradation of one enzyme is highly variable from one tissue to another. Thus, a study of LDH_5 in the rat reveals half lives of 1·6 days in the heart, 31 days in skeletal muscle and 16 days in liver. These figures contrast with figures of 1, 22 and 2·2 which are the half lives of the total soluble protein in the tissues (Fritz *et al.*, 1969). The steady state of LDH in any cell can, therefore, be increased or decreased by altering either the rate of synthesis or the rate of degradation.

A second fact, already alluded to in this section, is that different LDH isozymes may have very different degradation rates in the same tissues and so the electrophoretogram obtained from a tissue at any one time is also a reflection of differential degradation. This mechanism presumably provides an explanation for the incidence of the five isoenzymes in the rat kidney, where both LDH_1 and LDH_5 are strongly represented, while the intermediate hybrids, normally making up the bulk of the LDH of the cell, are present in lesser amounts. The possible difficulty of hybridization of the two A and B subunits cannot apply in this case since other tissues of the same organism give a more or less random polymerization of the subunits and the consequent prevalence of the hybrid tetramers. It is also likely that the breakdown of the A subunits in erythrocytes, leaving an absence of LHD_5 in these cells, is to be seen as a result of a contrived cellular control mechanism rather than accidental loss. Studies on LDH degradation in cells also permit another deduction. That is that the degradation is a first-order reaction. This implies that all molecules of the one species whether recently made or old have an equal chance of being degraded. In other words, no age-related ticketing system is apparent.

6. Comment

The contribution which studies of lactate dehydrogenase enzymes can and do make to our knowledge of gene expression closely parallels that of the haemoglobins. Both systems, offering changes during ontogeny, involve the products of only a few genes and these can be readily separated by electrophoretic methods. But LDH provides a supremely good system for studying the fine modulation of one or two cistrons in different tissues, an aspect not afforded by the haemoglobins. In addition, the pattern of LDH in cells and tissues is often the result of controlled enzymatic degradation as well as synthesis and, once more, comparisons between different tissues are immensely valuable.

It does seem very important that the factors affecting the levels of LDH in tissue should be carefully sorted out, so that the simple results of a purely genetic control, i.e. one gene determining the correct rate of transcription for another gene, be distinguished from cytoplasmic feed back to the genes in terms of metabolic demand for A or B subunit production. It is also necessary to work towards an understanding of how controlled degradation is engineered. Is it already tightly programmed by the genes or is it more casually modulated on a supply and demand basis? Since LDH enzymes are almost universally distributed and are by no means extraordinary proteins, they seem to be the safest and best model system in which to study rate control in gene expression, the finest and last phase of the genetic control of differentiation.

C. Haemoglobin

This topic will be discussed at slightly greater length than others in this book, partly because it is the system with which I am personally most familiar, but also because it is arguably the system about which more is known than any other in the field. Control mechanisms governing synthesis and accumulation of haemoglobin in blood cells have been studied at many different levels. These include comparative studies on the globins and haemoglobins of related organisms, haemoglobin synthesis in cell free preparations, globin gene multiplicity and the preparation of globin messenger RNA, haemoglobin synthesis in fused cells and in non-erythroid cells following haemoglobin messenger RNA injection, the role of the hormone erythropoietin and the synthesis of haemoglobin in tissue cultures of virus induced erythroleukaemic cells. Some of these topics are discussed in Section 4E on fused cells and message injection, but other details of these experiments will also be discussed in this chapter. A review on some aspects of gene expression in haemoglobin synthesis has already been written by myself and my colleague Richard Jurd (Maclean and Jurd, 1972).

1. Structure and distribution of haemoglobin

Haemoglobin, like lactate dehydrogenase and many other proteins, normally has a tetrameric structure, the molecule consisting of four subunits. Each subunit consists of a globin chain of some 150 amino acids, folded around a haem group which contains an atom of ferrous iron. Actually some haemoglobins exist as monomers, as for example in the primitive fishes, the Cyclostomes.

The structure of haem has been discussed by Harris and Kellermayer (1970). Haem is utilized in the formation of many other molecules besides haemoglobin, myoglobin, erythrocruorin, catalase, peroxidase and cytochrome B enzymes. This implies that iron labelling cannot be used for the detection of haemoglobin alone on a whole animal scale and even in very early stages in erythroid cell maturation, iron labelling may not be an accurate reflection of haemoglobin synthesis. Indeed all living cells capable of aerobic respiration can synthesize haem, and many organisms, for example most insects, possess haem in many tissues but do not synthesize any haemoglobin. Globin is found only in myoglobin and haemoglobin, and the globin chain sequence of myoglobin is normally quite different to any of the globins found in haemoglobin. Although myoglobin is found in most muscle cells, haemoglobin is normally confined to circulating blood cells and their precursors. Actually haemoglobin is to be found in a few other situations, e.g. in the root nodules of leguminous plants, in fungi such as *Saccharomyces* and *Neurospora* and in such protozoa as *Paramecium* and *Tetrahymena* (see review by Maclean and Jurd, 1972). However, these haemoglobins do differ slightly from those found in the blood cells of the vertebrate animals.

Haemoglobin in most vertebrates is, therefore, a protein which is, as far as can be detected by present methods, confined to the erythroid cells, although the haem portion of the molecule is also synthesized and utilized in many other tissues. Within the erythroid cells, different kinds of haemoglobins occur, this variation resulting entirely from variation in the amino acid sequence of the globins concerned. Not only do most adult vertebrates possess more than one type of globin, but they also possess more than one type of haemoglobin. The variation is often extended by the occurrence of different haemoglobins in the blood cells of the younger stages of the animal.

Let us first review the situation in man and then briefly compare the human situation with that found in other vertebrates. In the normal human adult the most abundant haemoglobin is termed haemoglobin (Hb) A, and consists of two α globin chains and two β globin chains making up the tetramer. All blood cells also possess another haemoglobin in much lower concentration, Hb A_2 consisting of two α and two δ chains. The proportions of A to A_2 haemoglobin remain very constant at 97·5% A and 2·5% A_2 (Kunkel and Walenius, 1955). If we examine the blood cells of the human foetus between three and six months of pregnancy, the haemoglobin will be found to be of a different type to the adult and is termed HbF, consisting of two α chains and two γ chains. In other words, at or about birth, the γ globin synthesis ceases and is replaced by β globin production. Synthesis of the α globin has continued throughout foetal and adult life. Going further back in development reveals yet other haemoglobins, the so-called embryonic haemoglobins, in at least five forms. These can be expressed as : $\alpha_2\varepsilon_2$, ε_4, $\alpha_2\zeta_2$. $\varepsilon_2\zeta_2$ and $\zeta_2\gamma_2$ (Capp *et al.*, 1970). Man, therefore, possesses three sequences of haemoglobins, embryonic, foetal and adult, of which all except the foetal occur as multiple forms.

Turning now to other vertebrates, many fish have differing haemoglobins in larval and adult life as well as multiple adult haemoglobins (Perez and Maclean, 1974, 1975a,b). So also do amphibians, where the tadpole haemoglobins normally differ from those of the adult, as reported for the bullfrog, *Rana catesbiana* (Moss and Ingram, 1965), the axolotyl, *Ambystoma mexicanum* (Maclean and Jurd, 1971a) and *Xenopus laevis* (Maclean and Jurd, 1971b). Multiple haemoglobins occur in birds also, together with ontogenic changes in the pattern of haemoglobins made, although in some species the changes consist in alterations in ratios rather than replacement of one band by another. The domestic fowl displays strikingly different patterns of embryonic and adult haemoglobins, however (Manwell *et al.*, 1966; Brown and Ingram, 1974). Mammals other than man also have ontogenic changes in haemoglobins, together with multiple adult haemoglobin forms. But some caution must be exercised in drawing comparisons since many small mammals, such as mice and rats, have no counterpart of the human foetal haemoglobin, an "embryonic" pattern giving place to an adult pattern at about day 12 of gestation (Shaw and Maclean, 1971).

Some points about these multiple haemoglobins and their succession in ontogeny require emphasis and comment. First, it should be noted that, in only a few cases, have the globin chains from the haemoglobins been isolated and identified. It, therefore, follows that many of the examples of multiple haemoglobins may be akin to the main isoenzymes of LDH, where many tetrameric forms may result from combinations of only two basic protein monomers. A new haemoglobin might be detected during development which resulted simply from an altered cytoplasmic environment in the red cell, favouring a new combination of previously existing subunits. Second, some species appear to have haemoglobin polymorphism within the group, one individual animal having a differing haemoglobin pattern from another member of the same species (see discussion in Manwell and Baker, 1970). Third, the question of whether these various haemoglobins can and do exist within the same cells is a very complex one and will be discussed shortly. Lastly, mutant forms of these varied haemoglobins are occasionally detected, as for example in the altered β chain of human haemoglobin A, which is found in individuals with the sickle cell gene. Such mutants are often of great value in genetic analysis and in studies on globin gene expression.

It is a pity that so many of the studies on the cellular and developmental distribution of haemoglobin have not involved determination of the globin chains involved. The reasons, however, are often legitimate. While the haemoglobin retains its haem group it retains its distinctive colour and so bands can be followed in chromatography and electrophoresis without staining or labelling. More significantly, haemoglobin molecules are normally stable in suitable salt solutions and will yield sharp and separate bands in a variety of separation methods. When haemoglobin is broken down into its separate globin subunits and the solution of subunits is electrophoresed (the bands are of course invisible and require staining) the bands of globin tend to be broad and diffuse and to run into one another. This is no doubt partly a result of not being able to assess the point of optimal separation—in the case of haemoglobin, since the bands are visible, a moment in time can be chosen for fixation when the bands are optimally separated. But it also seems to result from

instability of the globin molecule, so that a previously homogeneous population of such molecules can become slightly heterogeneous because of small alterations in the chain configuration. Since the single globin is the product of one structural gene, it is often difficult or impossible to make correct deductions about gene switching from studies on haemoglobins alone. In some cases the problem has been minimized by demonstrating that two haemoglobins, say a foetal and an adult haemoglobin, do not share antibody binding sites and, therefore, probably have no common globin chain (Maclean and Jurd, 1971).

Armed with this caution about interpreting globin distribution from haemoglobin studies, let us look now at the vexed question of cellular distribution of haemoglobins. If studies on changes in haemoglobin synthesis are to yield information about gene expression, it is obviously desirable that the change occurs within one cell, or at least within one line of cells. But this point is often in doubt and only in a few situations can one be certain that an intracellular change is involved. The situation is best understood by comparing two different situations. First, in the case of human foetal and adult haemoglobins, HbF and HbA, the evidence is very strong that one line of cells is involved and that, at or about birth, the switch occurs within individual cells. The best evidence for this assertion comes from babies carrying the sickle cell gene. This gene is a defective form of β globin which, together with α globin, makes up human HbA. Human foetal haemoglobin, it will be remembered, is a combination of α and γ globin. If the blood of such babies is examined at birth and the haemoglobins electrophoresed, it is found that they have a normal proportion of HbF. Nevertheless, if the blood is exposed to a reduced oxygen tension, all the corpuscles will sickle, revealing that HbS (the sickle form of adult haemoglobin) is present in every cell (Schneider and Haggard, 1955). The interpretation of this experiment is clear—that some cells possess both γ and β (S) globins and, therefore, that the haemoglobin transition is an intracellular event. Similarly, using a microelectrophoresis technique, human HbA and HbA$_2$ have been shown to co-exist in the same cell (Matioli and Niewisch, 1965), although an ontogenic change is not involved in their production.

In contrast to the HbF and HbS evidence in man is the picture which emerges for the ontogeny of mouse haemoglobins. Mice do not possess any true foetal haemoglobin, but change early in embryonic life from an embryonic pattern to an adult pattern.

Most of the available evidence clearly shows that the embryonic mouse haemoglobin is confined to yolk-sac-derived blood cells and the adult haemoglobin to the definitive (adult) red cells and that the two types of haemoglobin are not synthesized in any one cell (Marks and Rifkind, 1972). Even in the mouse, however, the evidence is not absolutely watertight. In my own laboratory one of my colleagues found that, when mouse yolk-sac-derived erythrocytes were kept in culture, they synthesized small amounts of mouse adult haemoglobin towards the end of their life (Shaw, 1969). This latter development may never occur *in vivo*, but clearly the genes involved are at least potentially active. The haemoglobin sequence and red cell development in the chicken is complex and has been fully discussed by Bruns and Ingram (1973).

The most difficult situation to interpret, perhaps, is that occurring in the lower vertebrates where a larval (or tadpole) haemoglobin gives place to an adult pattern at or about metamorphosis. In *Xenopus laevis*, Jurd and Maclean have found clear evidence that the transition from the tadpole to the adult pattern occurs within one line of cells, since both types of haemoglobin co-exist in some cells (Jurd and Maclean, 1970 and Jurd and Maclean, 1974). On the other hand, in studies on the American bullfrog (*Rana catesbiana*), Maniatis and Ingram (1971) found that the tadpole and adult haemoglobins did not co-exist in the same cells, again using immunofluorescence as a detection system. Single cell electrophoresis by Rosenberg (1970) on red cells from the same animal pointed to a similar conclusion. Adding to the confusion, however, is the recent finding by Benbassat (1974) that haemoglobins of both types do cellularly co-exist in this species.

The only satisfactory conclusion from this and much other evidence is that in some species the transition certainly involves an intracellular change; in other species it may not. Of course even in species where both foetal and adult haemoglobins can be clearly found within one cell, this cannot be taken to imply that in that cell foetal globin gene activity comes to be replaced by adult globin gene activity. In many cases the change over may involve exclusively adult or foetal production in some cells, and constant production of both in others. Actually the widespread incidence in the blood cell population of the minor haemoglobins at metamorphosis does tend to favour an intracellular change in gene expression at the time of transition (Jurd and Maclean, 1970). But it must be emphasized that in no case has it been shown that the intracellular controlling event is a transcriptional one, nor that one gene is active at one time in the life of the cell and another globin gene at another. Such studies would be most useful but none have, to date, been reported. A hypothesis widely favoured to explain the change in haemoglobin pattern involves the maturity of the red cell and its previous mitotic experience (Baglioni, 1963).

The hypothesis that the pattern of genes expressed by a cell may be affected by its mitotic maturity, i.e. the number of cell divisions between the stem cell and differentiated end cell, also finds a possible application in studies of foetal haemoglobin production in the anaemic or stressed adult. For example in the human, substantial production of foetal haemoglobin may be induced in adult life by sudden anaemia, or even by pregnancy (Horton and Huisman, 1965). Similarly, tadpole haemoglobin is synthesized by adult *Xenopus* when they are rendered anaemic by blood removal or phenylhydrazine injection (Maclean and Jurd, 1971b). In these examples the foetal or tadpole haemoglobin is widely distributed throughout the erythrocyte population and, therefore, clearly co-exists with the adult form. As we will discuss later in this section, erythropoietin levels may have some role in the induction of the foetal/tadpole haemoglobin production, but this may also operate through an effect of the mitotic experience of the cell.

Despite the intensive research on haemoglobin and the many fascinating changes in the synthetic pattern recorded, in no case is the relationship to transcription really clear. Thus, although changes in haemoglobin synthesis are widely quoted as examples of gene switching during development, we cannot as yet be certain of the events within any one red cell. If it can be shown that the foetal and adult haemoglobins are synthesized together or in sequence within any one cell, that would be an important step forward, although even that knowledge would not rule out post-transcriptional control as the *modus operandi*.

2. Erythroid cells

One of the great attractions of the haemoglobin system is the exclusive production of this protein within the red blood cells and their progenitors. As we have commented earlier, it is actually the globin which is unique to these cells, since haem is very commonly synthesized in almost all cells. Although globin exists in the myoglobin of muscle, it is a distinct form of the molecule, although no doubt the globins of myoglobin and haemoglobin have an evolutionary connection in the evolution of the genome (Ingram, 1963). The evidence that the globin of haemoglobin is confined to red blood cells is not, in fact, absolutely watertight. It would be a most rewarding but demanding task to screen other cell types for indications of low level globin production. Since available probes, such as anti-haemoglobin sera conjugated with a fluorescent molecule, give apparently non-specific binding on some other cells, the search probably demands better assays than are currently available.

Erythroid cells occur in almost all vertebrates. Larval eels and the ice fish of the antarctic lack haemoglobin and have, therefore, no proper red blood cells (Ruud, 1954) and in some invertebrates the haemoglobin is free in the plasma and not contained within cells (see review by Maclean and Jurd, 1972).

Erythrocytes, together with granular leucocytes, originate in vertebrate myeloid tissue and are often termed myeloid elements. Such tissue is also called erythropoietic tissue. In adult mammals, the red regions of bone marrow are the main erythropoietic sites, while vertebrae, sternum, ribs, the diploe of the skull bones and the proximal epiphyses of femur and humerus all contain red marrow.

In the vertebrate embryo, erythropoiesis occurs at sites different from those active in the adult. In the early mammalian embryo, some of the yolk sac cells give rise to the blood islands of primitive or embryonic red blood cells. Such primitive cells often retain their nuclei. During development other tissues, such as pronephros, liver, lymph nodes, spleen and bone marrow supplant and finally replace the yolk sac as erythropoietic sites. The picture varies somewhat in the lower vertebrates, with organs such as liver and spleen remaining as important erythropoietic sites throughout adult life.

The mature red blood cells of adult fish, amphibia and birds retain their nuclei, while those of mammals are anucleate. In both the lower and higher vertebrates the myeloid (erythropoietic) elements and the lymphoid elements are often mixed and probably may have common stem cells, at least in embryonic development. The existence of lymphoid cells which are capable of haemoglobin synthesis in the Friend leukaemia of mice (Friend, 1957) seems to emphasize this relationship—see later discussion in this section.

In both embryo and adult the red blood cells arise from relatively undifferentiated stem cells and indeed such cells may be common to the myeloid and lymphoid cell lines (Bloom and Fawcett, 1968). Erythroid stem cells are referred to as haemocytoblasts. These cells are amoeboid, basophilic and have non-granular cytoplasm and are indistinguishable from many lymphocytes. The developmental series, derived from the haemocytoblast, is haemocytoblast–proerythroblast–basophilic erythroblast-polychromatophilic erythroblast–normoblast–reticulocyte–erythrocyte. Haemoglobin synthesis can normally be detected first in late basophilic erythroblasts. An excellent experimental system which permits determination of erythroid stem cell numbers has been developed by Till and McCulloch (1961). The technique involves injection of mouse cell suspensions, derived from presumptive erythropoietic sites, into lethally irradiated mice. The stem cells establish clones within the host spleen (Becker *et al.*, 1963) and the erythroid nature of some of the clones can be determined by sectioning of the spleen and examination of the clonal cells. A discussion of the exploitation of this technique in studies on mouse erythropoiesis will be found in Cole (1975). The spleen colony-forming cell is, in fact, the multipotent stem cell, and gives rise to clones of potential megakaryocytes (platelet progenitor cells), erythroid stem cells, granulocyte stem cells, or lymphoid cells. All these separate cell lineages are derived from a common stem cell, at least in the mouse. Mammalian adult reticulocytes and erythrocytes are annucleate, the reticulocytes being so called because of the persistance of a cytoplasmic reticulum, stainable by brilliant cressyl blue and other stains, representing the remains of the cell membranes present in the normoblast. Non-mammalian vertebrates retain the cell nucleus even in the erythrocyte and the term reticulocyte has less relevance in these animals (Maclean *et al.*, 1969).

The existence of different islands of erythropoietic tissue implies the existence of an erythropoietic archipelago in most vertebrates, but the developmental significance of this is not always clear. Whether, for example, the yolk sac of the mammalian embryo actually seeds the other erythropoietic sites, or whether they arise independently, is not certain. This has led to considerable confusion in the literature about the existence of two distinct morphologically distinguishable populations of cells, synthesizing the embryonic and adult haemoglobins

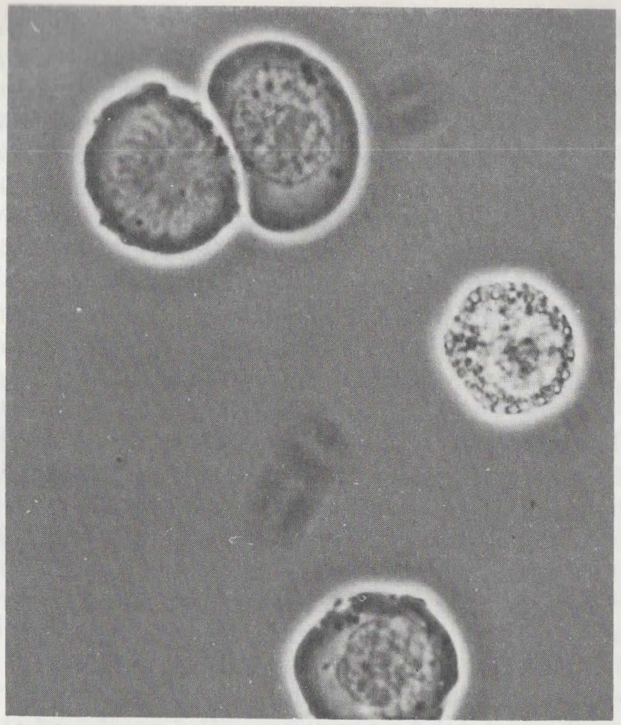


FIG. 5. Living cells photographed by phase-contrast microscopy from the blood of an anaemic *Xenopus*. The animal had been rendered anaemic by an injection of phenylhydrazine 15 days prior to bleeding. These cells are typical of the blood cells at this stage, one being a granulocyte and the others erythroid cells early in differentiation. One erythroid cell is seen in mitosis.

(Fantoni *et al.*, 1968) or of no correlation between erythroid cell populations and the distinct types of haemoglobin (Fraser, 1964). The situation may vary in different organisms, the first paper quoted involves work on the mouse and the second on the chicken. But even in one organism, say the mouse, it is not clear from the literature whether the two populations are distinct and have essentially separate developmental origins, whether one seeds the other or whether they are essentially the same populations of cells with massive migration from one island site to another (Barker, 1968).

One of the considerable disadvantages of the haemoglobin system for studies on gene expression must now be obvious to the reader. It is that, although the circulating blood cells represent a relatively homogeneous cell population rich in one particular gene product, globin, the actual synthesis of that protein largely occurs before release of the red cell to the circulation. This fact represents a grave disadvantage, since all the crucial steps in the mechanics of gene switching occur in the erythropoietic tissues which are far from homogenous and constitute a very difficult experimental material. One way to alleviate this difficulty would be tissue culture of the myeloid elements, but they are often rather resistant to satisfactory tissue culture. Culture of mammalian erythroid cells *in vitro* is comprehensively discussed by Axelrad *et al.* (1974). Modern approaches to this problem involve culture in semi-solid media such as agar and have permitted analysis of the sensitivity of the erythroid cells to erythropoietin and "colony stimulating factor" (Gregory *et al.* 1973). An alternative is to experiment with profoundly anaemic animals since, in such animals, many of the early differentiating steps in the haemoglobin synthesis pathway occur in the circulating cells (Thomas and Maclean, 1974; Thomas and Maclean, 1975; Hilder *et al.* 1975; see Fig. 5) and such cells may be maintained in tissue culture at least for the duration of differentiation. I believe that studies on the control of haemoglobin synthesis in the blood cells of such highly anaemic animals are likely to be very productive in terms of increasing our knowledge of how gene expression is controlled. The fact that anaemic *Xenopus* are also synthesizing some tadpole haemoglobin in their blood cells is, of course, an added bonus.

Another promising line of research in erythropoiesis involves chalones, tissue specific regulators of cellular proliferation presumed to be released by the appropriate tissue cells themselves. A recent review of chalones has been undertaken by Thornley and Laurence (1975). An erythrocytic chalone was reported by Kivilaakso and Rytömaa (1971) and confirmed more recently by Bateman (1974).

3. Control of haemoglobin synthesis

The levels at which gene expression may be controlled are more fully discussed in Chapter 1 of this book. They are briefly either transcriptional, which covers control over whether or not a gene is transcribed and at what rate, or post transcriptional, which includes many different steps such as modification, selection and selective breakdown of messenger RNA, cytoplasmic availability or storage of latent mRNA, rate of ribosomal handling of the mRNA and of the release of the completed polypeptide chain from the ribosome and the controlled degradation of the final protein product. As has been emphasized above, in most of the examples of changes in haemoglobin synthesis, the precise mechanisms and levels of control are not known. It is in most cases a matter of monitoring the protein and its rate of synthesis and hopefully extrapolating back to a gene control mechanism. To the extent that some control mechanisms are understood for haemoglobin synthesis, however, we will now discuss the available knowledge.

(a) *Synthesis of haem and its effect on haemoglobin synthesis*

As discussed by Maclean and Jurd (1972), the main control mechanism affecting haem production is in fact the first step in the pathway of its synthesis, the production of δ-aminolevulinic acid (ALA). The production of ALA is in turn governed by an enzyme δ-aminolevulinic acid synthetase (ALA synthetase) and it seems probable that rate control over manufacture of this enzyme is the master switch governing haem synthesis. Other factors are also involved and no doubt vary in importance from one organism to another.

ALA synthetase is a mitochondrial enzyme, but is coded on the nuclear genome and manufactured on cytoplasmic ribosomes (Scholnick *et al.*, 1969). There are now good grounds for believing that the primary product is an inactive form of the enzyme and that activation of the enzyme within the mitochondrion occurs, perhaps by shearing off part of the original protein (Tuboi and Hayasaka, 1971). In some organisms it seems probable that the glycoprotein hormone erythropoietin can stimulate haem synthesis, e.g. in yolk-sac-stage embryonic mouse cells (Bateman and Cole, 1971). These effects will be discussed further under the heading of Erythropoietin (see page 70).

Completion of haem, including insertion of the iron into the protoporphyrin, apparently occurs within mitochondria. Haem is then released into the cytoplasm. An important question then arises, namely does the availability of haem affect the rate of globin production? Burnham (1969) has presented an interesting scheme whereby haem would promote release of globin chains from the polysomes. The effect remains somewhat speculative, however, since labelled haem has not yet been shown to bind selectively to the polysomes involved in globin assembly. Indeed there are still conflicting views on whether or not haem and globin combine while globin is still attached to the polysome or after release of the globin (see Discussion in Maclean and Jurd, 1972). Addition of free haem undoubtedly increases the rate of globin synthesis in a cell free system (Hunt *et al.*, 1972), but this might be due to the influence of free globin on its own synthesis. Some very dramatic effects of haem (or haemin) on the translation of globin mRNA have recently been revealed. Giglioni *et al.* (1973) have shown that, when haemin was added to oocytes which were also injected with mouse globin mRNA (see discussion in Section 4E), the ratio of synthesis of α to β globin rose from about 2 : 10 to almost 1 : 1. Since both globin mRNAs are translated at about equal efficiency in a reticulocyte cell-free system (Gurdon *et al.*, 1974), the addition of haemin to the oocyte may be interpreted as making up the oocyte cytoplasm to the equivalent of the reticulocyte. In both Giglioni's and Gurdon's experiments, the injected mRNA was believed to be about 50/50 mixture of α and β globin mRNA. As Gurdon comments (Gurdon, 1974), haemin seems not only to be a factor which affects messenger translation but it does so in a selective and message-specific manner. Curiously enough, although these results are valid for mouse globin mRNA, injection of rabbit globin mRNA gives a 1 : 1 ratio of α to β globin, without added haemin, in situations where the mouse globin mRNA without haemin gives poor α globin synthesis (Gurdon *et al.*, 1974).

Actually, it has been shown that ribosomes bind to β globin mRNA more efficiently than to α globin mRNA, perhaps because the former is longer and forms larger polysomes (Lodish, 1971). The effect of haemin may, therefore, be on initiation of the globin chains, as suggested by Hunter (1974). Curiously enough, haem affects the rate of synthesis of some non-haem proteins in reticulocytes (Lodish and Desalu, 1973) and may perhaps do so in other cells as well. It may be that haem interferes specifically with an inhibitor of chain initiation for globin, and for many non globin proteins, and such a role has been discussed by Hunter (1974).

(b) *Message selection and nuclear RNA*

Since the discovery of very high molecular weight RNA inside the nuclei of many eukaryotic cells, including cells of the erythroid series such as immature duck erythrocytes (Attardi *et al.*, 1970), many people have suggested that such RNA might consist of multiple copies of mRNA. Processing of such RNA within the nucleus would then constitute an important level of control over gene expression. Although there does seem to be sound evidence for intranuclear degradation of nuclear RNA (Harris, 1970), recent experiments do not suggest that this nuclear RNA consists of repeated copies of mRNA. But the existence of some copies of the globin mRNA in the heterogeneous nuclear RNA of embryonic mouse liver has been demonstrated by Williamson and Drewienkiewicz (1972) and Williamson *et al.* (1973).

The prevalence of high molecular weight RNA in both duck erythrocytes (Attardi *et al.*, 1970) and chicken erythrocytes (Maclean and Madgwick, 1973) seems to confirm that this species of RNA is not particularly associated with rapid cellular metabolism and is not primarily a mRNA precursor molecule. Lewin (1974b) has made some interesting calculations on the frequency of globin sequences in the Hn RNA and on the proportion of Hn RNA carrying globin gene sequences in reticulocytes of the duck. Using data from the experiments of Williamson *et al.* (1973) and Imaizumi *et al.* (1973), Lewin calculates that 0·3% of the Hn RNA corresponds to globin mRNA and that between 7 and 52% of Hn RNA molecules contain a globin sequence. Many assumptions are of course inherent in these calculations and the high variability of the answer simply reflects the different methods of preparing the Hn RNA. If each Hn RNA molecule can contain only one globin gene, then 52% would be expected to carry the gene.

Most of the Hn RNA is not normally permitted to pass to the cytoplasm but is degraded within the nucleus (Shearer and McCarthy, 1970).

(c) *Transfer RNA*

The role of transfer RNA in cell differentiation has been reviewed by Sueoka and Kano-Sueoka (1970). Although it is clearly possible for availability and variable synthesis of tRNA to exert control over protein synthesis at the level of translation, there are no strong grounds for believing that it does so commonly. But some scattered evidence does suggest that alterations in the population of tRNA molecules occur as an accompaniment to differentiation. For example, one of five aminoacyl-tRNAs examined showed a large difference in relative amount as between red cells of the chick embryo and the erythrocytes of the adult bird (Lee and Ingram, 1967). One particular isoacceptor form of tyrosine tRNA functions as an inhibitor of tryptophan pyrrolase in the vermilion mutant of Drosophila (Jacobsen, 1971), so clearly tRNA molecules could have regulatory roles in gene expression in particular cells or organisms.

It is possible to be fairly certain that tRNA molecules are reutilized a number of times. This can be calculated from fairly simple data discussed by Maclean and Jurd (1972). The calculation assumes that 150 amino acids are incorporated into a single polypeptide in three mins at 37°C in a eukaryotic cell and that, on a mass for mass basis, each ribosome would have an available stock of 1000 tRNA molecules. Such figures imply a requirement for each tRNA molecule every 20 mins. Since transfer RNA synthesis is not rapid, even in nucleated erythrocytes (Maclean and Madgwick, 1973) and cannot happen at all in the mammalian reticulocyte, recirculation of the molecule seems to be inevitable in globin production.

(d) *Ribosomal RNA*

There is no obvious way in which ribosomes or ribosomal RNA production can selectively affect the types of protein manufactured by a cell. At the moment, almost all the available evidence suggests that ribosomes are not specific for the proteins whose production they mediate. Nevertheless, in a cell such as an erythrocyte, there is a controlled decline in productivity with maturation and Attardi *et al.* (1970) have shown that this is accomplished in the duck erythrocyte partly by reducing the rate of ribosomal RNA synthesis. It also seems likely that this reduction in rate of synthesis may be accompanied by an increased rate of specific degradation of the cellular ribosomes.

(e) *Messenger RNA*

The literature on globin messenger RNA is now voluminous, largely because this species of mRNA has proved to be the easiest to prepare and has indeed been isolated from mouse reticulocytes in milligram quantities (Williamson *et al.*, 1971). The mammalian reticulocyte cell free system has also been very popularly used to study the kinetics of translation of endogeneous and exogenous mRNA. Globin genes have also been synthesized by the reverse transcription of 10S rabbit globin mRNA using viral reverse transcriptase enzyme (Verma *et al.*, 1972). We will only discuss here the ways in which the messenger RNA for globin can or does effect control over globin synthesis. Present knowledge on the subject is best discussed by itemizing each point separately.

1. Messenger RNA has been isolated from a number of different cell types such as eye lens, muscle, silk gland and plasma cell, but the globin mRNA from mouse, rabbit and duck reticulocytes has been the type most intensively studied.
2. Although total reticulocyte RNA did not at first give signs of producing globin synthesis in a bacterial cell free system, provision of 8S rabbit reticulocyte RNA plus N-acetyl-valyl-tRNA has produced rabbit globin synthesis in such a bacterial system (Laycock and Hunt, 1969).
3. Fewer ribosomes are associated with the α than the β globin mRNA. Original suggestions that the translation rates are also different is probably incorrect (Hunter, 1974).
4. Haem affects the rate of translation of globin mRNA and sometimes the translation of α and β globins differently. It may also affect the translation rates of non haem proteins (Hunter, 1974 and Gurdon, 1974).
5. The amount of globin mRNA in reticulocytes does not itself appear to be rate limiting (Lingrel *et al.*, 1971) in the cell free system, but *in vivo* added globin mRNA leads to increased globin production in message injected animals (Moar *et al.*, 1971).
6. In many stages of development and in the adult reticulocyte, the half life of globin mRNA is very long. In Gurdon's message injection experiments, rabbit or mouse globin was still being synthesized many days after injection of the mRNA into fertilized *Xenopus* eggs, and a half life in excess of eight days was indicated (Gurdon *et al.*, 1974). However, its life span is probably very dependent on specific mRNA degradation. Anaemic chicken red blood cells demonstrate Actinomycin D sensitivity of leucine uptake within half an hour of incubation and at ten h the incorporation of leucine into protein (of which most is globin) is halved (Maclean and Madgwick, 1973). It seems fair to conclude that globin mRNA is potentially very stable and is itself probably controlled by specific degradation.
7. *Xenopus* eggs, following injection of globin mRNA, grow into tadpoles and frogs in a relatively normal manner. When the tadpoles from such eggs were examined and the distribution of rabbit or mouse globin assessed, as much heterologous globin was found in non-erythropoietic tissues as in the blood cells and their precursors (Woodland *et al.*, 1974). Moreover, where alterations in the ratio of α to β globin synthesis occurred, these alterations were apparent in both erythropoietic and non-erythropoietic tissues.

8. Using a complementary DNA probe made against globin mRNA by viral reverse transcriptase enzyme, Chan *et al.* (1974) have assayed cells of developing chicks for globin mRNA. They have shown that the existence of masked mRNA prior to globin production is unlikely, since a surge of globin mRNA production coincides with the burst of globin synthesis. They also suggest that a chick embryo pro-erythroid cell has less than 300 globin mRNA molecules, while the differentiated chick erythroid cell possesses about 10^4 such molecules. Ross *et al.* (1974) suggest a figure of 7–8000 globin mRNA molecules in the mouse reticulocyte.
9. The role of the polyadenylate-rich sequences at the 3′ terminus of mRNA has roused interest. There is some experimental evidence that these segments become shorter with the age of the molecule (Shieness and Darrell, 1973), leading to a suggested ticketing system exerting control over the life span of the molecule. Huez *et al.* (1974) have compared translation rates of rabbit globin mRNA with and without the poly A sequence, following its injection into Xenopus oocytes. Initial translation rates were similar, but a greater fall-off in translational efficiency occurred with the mRNA without poly A sequences. It, therefore, seems probable that the poly A sequence of globin and other mRNA species is not necessary for faithful translation, but does stabilize the molecule so that it can withstand repeated translation. Such an interpretation would permit a ticketing system, as suggested above, although it does not, of course, prove that the system operates in that way.
10. As mammalian erythroid cells develop, haemoglobin synthesis becomes independent of further RNA synthesis and this stabilization affects cells at many different developmental stages in a more or less synchronous manner (Djaldetti *et al.*, 1970). This suggests that globin mRNA production in cells at various stages may be commonly sensitive to a generalized stimulus, perhaps hormonal.
11. There has been considerable speculation about the possibility that native messenger RNA, when operating *in vivo*, is complexed to protein and, therefore, that the true messenger molecule is a ribonucleoprotein. Any attached protein would normally be removed by the isolation procedures used for

mRNA preparation in most laboratories. However, a particularly active cell free system has recently been employed to assay globin mRNA with and without associated protein (Freienstein and Blobel, 1974). Virtually no difference in the rate or fidelity of translation was detected. Many experiments on transcriptional regulation involve reconstituted reticulocyte chromatin and probes for globin mRNA. This work is discussed in Section 7A.

(f) *Post transcriptional control by globin on its own synthesis*

There are two aspects to this topic, both of considerable interest. The first is that α globin has been shown to regulate its own synthesis in a cell free system, apparently by end-product inhibition (Blum *et al.*, 1970). Such a mechanism must ensure a constant pool size for free α globin. The second aspect is the demonstration by Fischer *et al.* (1968) that β globin is only released from the polysomes in the presence of free α globin, presumably through α/β dimer formation. Apparently globin dimers leave the polysomes and combine in the cytoplasm to yield the functional tetramer. Whether the haem associates with the globin before or after release from the polysome is, as we have previously commented, uncertain.

(g) *Erythropoietin*

This substance, or group of substances, is probably a glycoprotein and has hormonal properties which seem to be specific to erythropoietic tissues. Reviews on the subject of erythropoietin will be found in Lange and Pavlovic-Kentera (1964) and Gordon (1971). Since it is a fairly large molecule, it is presumably questionable whether erythropoietin (EP) enters the affected cell; it may operate as do many other polypeptide hormones by a cell surface activation of adenyl cyclase and the second messenger system of cyclic AMP. The effects of EP on erythroid cells are diverse and probably vary in different organisms. Some degree of species specificity for EP is apparent (Gordon, 1971). There is good evidence that EP stimulates haem synthesis by erythroid cells *in vitro* (Bateman and Cole, 1971) and also the rate of cell division in erythropoietic tissues (Paul *et al.*, 1973). The latter authors also find that erythroid cell production by cultures of foetal mouse liver cells involves fewer cell divisions between erythroblasts and later erythroid stages if EP was added. In other words cell maturation would be accomplished with fewer divisions. We have already suggested in this section (page 59) that such a process might itself influence the particular globin genes expressed by a cell. Increased RNA synthesis in early erythroid cells is also commonly observed following EP treatment of cell cultures (Djaldetti *et al.*, 1972), sometimes showing some dependence on a prior burst of DNA synthesis (Cole and Paul, 1966). Perhaps the most important observation made on the effect of EP, one emphasized by Djaldetti *et al.* (1972), is that only some of the cells in the erythropoietic cells are sensitive to the hormone and they seem to be already partly differentiated erythroid cells. The erythroid stem cell and the differentiated reticulocyte or erythrocyte have little or no capacity to respond to the hormone.

It is highly likely that EP is mainly or entirely responsible for the response of an animal to anaemia. Whether it is also chiefly responsible for day to day regulation of erythropoiesis and haemoglobin synthesis is more doubtful.

Other hormones, particularly thyroxine and prolactin, do influence erythropoiesis in some organisms, especially during metamorphosis, but we will not discuss their role here. They are discussed by Maclean and Jurd (1972) in this connection.

4. The erythrocyte as a terminally differentiated cell

The erythrocyte is often quoted as the prime example of a terminally differentiated cell and certainly the anucleate mammalian erythrocyte is perhaps scarcely deserving of the title of cell. However, the nucleated erythrocytes of lower vertebrates are less extremely terminal, although highly specialized, and indeed the levels of RNA and protein synthesis in these cells are higher than is sometimes supposed (see discussion in Maclean and Jurd, 1972). Nevertheless, the erythrocytes of amphibians, in particular, provide us with an example of a relatively inert nucleus, with highly condensed chromatin. We are attempting in my own laboratory to study gene activation and repression in such nuclei by exposing preparations of isolated nuclei to ionic solutions and cytoplasmic fractions capable of reactivating RNA synthesis (Hilder and Maclean, 1974).

5. Erythrocyte included in fused hybrid cells

As is now well known, chicken erythrocytes and their nuclei have been included in heterokaryons and assays undertaken for the detection of globin synthesis. The general results obtained in these heterokaryon experiments are discussed in Section 4E of this book. Two particularly relevant aspects of these experiments are worth picking up here. The first is that reactivation of chicken erythrocyte nuclei has been reported (Harris, 1970a,b) when these nuclei are included in a heterokaryon along with a HeLa cell or rabbit macrophage. The findings for RNA synthesis must be viewed with some caution, since these nuclei already do synthesize RNA (Madgwick *et al.*, 1972), it would certainly be much more impressive to find substantial RNA synthetic activity in a *Xenopus* erythrocyte nucleus included in a similar heterokaryon. When included in a heterokaryon with a HeLa cell, the chicken erythrocyte synthesizes DNA, a phenomenon which does not arise in the rabbit macrophage/chicken erythrocyte combination (Harris, 1970). Neither of these latter cell types normally synthesize DNA, but since the chicken erythrocyte nucleus is in the G_1 phase of the cell cycle, presumably the synthesis in the HeLa heterokaryon represents the normal process of DNA replication and so indicates true reactivation. That such highly condensed chromatin is still capable of going through replication, albeit after nuclear swelling and chromatin decondensation, is itself remarkable.

The second aspect of the heterokaryon experiments which deserve comment here is the evidence about globin and haemoglobin synthesis in chicken erythrocyte and mouse fibroblast heterokaryons. Although chicken-specific protein synthesis has been recorded in these heterokaryons (a hen-specific cell-surface antigen and a soluble enzyme, inosinic acid pyrophosphorylase, was detected (Harris *et al.*, 1969; Harris and Cook, 1969), no reactivation of haemoglobin production has been observed (Harris, 1970). When the heterokaryons included only a chicken erythrocyte nucleus, no haemoglobin synthesis was ever detected, but when erythrocyte nucleus and cytoplasm (from three- to five-day-old chick embryo erythrocytes) were included in the hybrid cell, an increase followed by a gradual decline of haemoglobin synthesis was observed, becoming undetectable on the fourth or fifth day. The brief increase in haemoglobin production is no doubt a response to the sudden availability of a large pool of amino acids and haem. It should be noticed that these experiments do not suggest that the chicken globin mRNA is very long lived in these cells (see discussion on mRNA in this chapter). Harris' interpretation of the results (Harris, 1970) is that the recipient fibroblast cell possesses repressor proteins against its own globin genes and that these become effective against the globin genes of the chicken erythrocyte. Some recent experiments with hybrid cell lines do show considerable variation in the pattern of genes expressed, including the induction of protein not previously synthesized in either cell before fusion (Peterson and Weiss, 1972).

6. Haemoglobin synthesis by erythroleukaemic cells

A remarkable mouse leukaemia was discovered and developed by Friend (1957). The leukaemia is induced by the Friend leukaemia virus in susceptible mouse strains and presents in the affected mouse as erythroblastosis and spleen and liver enlargement some two weeks after inoculation with virus. Affected mice die after about two months and are found to have massive infiltration of spleen and liver by leukaemic cells. Tissue culture lines of such leukaemic cells have been established (Farusawa *et al.*, 1971) and are now maintained in culture.

Not only does this leukaemic cell line serve to underline the relatedness of lymphoid and erythroid cells, but it has proved to be a very valuable line of cells for studies on haemoglobin synthesis. This is chiefly because the cell cultures of the leukaemic cells make variable amounts of haemoglobin and some synthesize no detectable haemoglobin (Ross *et al.*, 1974), but production of the protein can be initiated or stimulated by exposure of the cell cultures to dimethyl sulphoxide at 0·5–2% concentration (Friend *et al.*, 1971). Recent studies of these cells include Sendai-virus induced fusions between cells of different erythroleukaemic cell lines, in order to determine the genetic and cytoplasmic influences on haemoglobin production (Paul and Hickey, 1974).

Apart from remarking on the obvious usefulness of these cell lines for studies on gene expression in erythroid cells, we should also discuss the curious action of the compound dimethyl sulphoxide (DMSO). This drug has been found to cause a large increase in the rate of erythroid maturation and of haemoglobin and haem synthesis in Friend leukaemia cell lines (Friend *et al.*, 1971). Unfortunately the mechanism of action of the drug is not, as yet, understood. It does affect non-erythroid cells in that it inhibits the blastogenic transformation of lymphocytes following exposure to phytohaemagglutinin (Mangi and Mardiney, 1970).

7. Globin gene frequency in the genome

Since globin is manufactured in very large amounts by erythroid cells, the globin genes have been suspected of being present as repeat copies on the genome, or perhaps specially amplified in early erythroid cells. Moreover, the general concept of multiple gene copies, especially in combination with a master/slave hypothesis (Callan, 1967), requires an experimental system in which bulk homogeneous mRNA would permit hybridization studies and a possible resolution of the problem. All the present evidence suggests that probably less than ten globin genes exist in the genome of the duck (Bishop *et al.*, 1972) and the mouse (Ross *et al.*, 1974). At least two copies of a gene for a single globin chain may, however, exist and evidence for this in the case of human α globin has been obtained. Other evidence relevant to this topic is discussed by Maclean (1975).

There is no experimental basis for the suggestion that gene amplification—the production of extra gene copies of a particular cistron in one differentiated tissue—occurs in erythroid cells. We must, therefore, conclude that the extreme specialization of the erythroid cell and its massive and exclusive production of globin depends on the mechanism beyond the primary gene level, since only one or perhaps two copies of a gene exist for each type of globin synthesized. The chromosomal locations of the genes for globin (including myoglobin) suggest an evolutionary scattering from a common single locus (Ingram, 1963). The genes for human β and α chains are closely linked.

8. Comment

It is obvious that a great amount of knowledge now exists concerned with haemoglobin and the control of its production. Each globin is the product of one, or at the most, two or three genes and no gene amplification is involved in the commitment of the erythroid cell. Globin gene sequences exist in the high molecular weight nuclear RNA, but the bulk of this RNA in erythroid cells has some other function than coding for globin. The rates of ribosome association with differing globin mRNA molecules vary, but the actual translation rates do not. Haem is in some way concerned with the initiation step of globin mRNA translation and perhaps with that of other mRNA molecules also. A pool of α globin affects the rate of α globin release from polysomes and α globin affects the release of β globin, probably by dimer formation. Some globin mRNA is long lived, with a half life of 20 days or more, but in embryonic or the immature blood cells of anaemic animals its turn over is much faster, often being measured in hours. The polyadenylate sequences of the globin mRNA are not essential to its translation, but affect the mRNA survival. Globin, of the haemoglobin type, is not apparently made normally in non haemic cells, but messenger RNA injection experiments reveal that such cells will freely translate such mRNA if it is made available to them.

Such is a reasonable summary of our present knowledge. What remains to be determined? In answering this question, the real state of our ignorance about genetic control mechanisms in eukaryotes becomes apparent, since the control of globin gene activity remains a closed book. Ignorance persists over how globin genes are silenced in non erythroid tissues but expressed in the blood cells and their progenitors and also how changes in globin gene activity during development or adult adaptation are engineered. The notion that the mitotic history (i.e. the numbers of mitosis experienced) of an erythroid cell may determine which globin genes are expressed, and in what order, remains popular but unproven. It is particularly evident in studies of haemoglobin synthesis that theories about gene activation are largely based on inference from information about when particular proteins can be detected. Probably, much of the persisting ignorance about gene activity and regulation in erythroid cells has resulted from the relative inaccessibility of the cells within which the gene modulation occurs. Studies on erythroleukaemic cells in culture and on the circulating precursor cells of profoundly anaemic amphibians should serve to overcome this hurdle in future. Similarly, the nucleus of the erythrocytes of lower vertebrates, by dint of its extreme genetic shutdown, may prove particularly useful for our understanding of the mechanics of gene repression. Further study of the excessive or reduced production of α or β globin chains in different human thalassaemia's should also prove rewarding in terms of understanding gene modulation (see review by Stamatoyanmopoulos, 1972).

D. Lens crystallins

A review on differentiation in vertebrate lens and a discussion of its usefulness in studies on gene expression will be found in Clayton (1970). In many ways the system is very similar to that of erythroid cells, in that a relatively homogenous population of cells becomes highly differentiated, even to the eventual loss of the cell nuclei, and their differentiation is accompanied by the accumulation of a group of proteins not found in other tissues—the crystallins. Other particular advantages of the vertebrate lens system are its immunological isolation (thus rendering its constituent proteins highly immunogenic), its regeneration from a variety of eye tissues following lentectomy and its differentiation *in vitro* from cultured cornea.

The vertebrate lens is composed largely of lens fibre cells which eventually become anucleate when mature and are then very rich in the characteristic lens protein, crystallin. A useful account of the normal growth and development of the lens is given by Truman (1974). The lens is derived in development from the ectoderm, following stimulation of the ectoderm by the adjacent optic cup. The early lens consists of a vesicle of epithelial cells and, with time, some of these epithelial cells elongate and come to occupy the central lumen of the lens vesicle These are the primary lens fibres. Throughout subsequent life the remaining epithelial cells which lie along one side of the lens continue to produce differentiation cells, by division, which form the successive layers of secondary lens fibres surrounding the primary fibres. Lens fibre differentation from dissociated cells of chick lens epithelium has been observed *in vitro* (Okada *et al.*, 1971), as judged both by synthesis of the lens-specific protein delta crystallin and by the appearance of the cells in the electron microscope.

Studies on vertebrate lens are relevant to questions of gene expression in a number of quite separate ways and we will, therefore, discuss the system under these separate headings.

1. Regulation of crystallin synthesis

Crystallins have been isolated from many vertebrate lenses and separated on the basis of molecular weight and electrophoretic mobility. Most can be separated into the α, β and γ crystallins, the α crystallins being those of highest molecular weight and mobility, γ crystallins the lowest and the β crystallins are intermediate. All classes of crystallins are highly heterogeneous. No specific enzyme function has been ascribed to these proteins but they have an important structural role in determining the refractive index of the particular lens area in which they occur.

Unfortunately, these proteins tend to exist in dynamic equilibrium with their subunits, making determination of *in vivo* quaternary structure very difficult (Clayton, 1970). Crystallins are, therefore, most frequently separated and studied in dissociating conditions, such as 8 M urea, so that it is the subunits and not the aggregate molecules which are studied. But even at this level, it has not proved easy to determine how many separate polypeptide chains and, therefore, how many separate genetic loci are involved in crystallin synthesis. The precise number of polypeptide subunits in the α, β and γ crystallins apparently varies in different species and is certainly multiple for each, with a number of major and minor bands appearing in electrophoretic separation. The multiplicity of crystallin monomer chains is something of a disadvantage in terms of using the lens system for the study of gene expression. From this point of view the globin chains of haemoglobins are often simpler. Most animals appear to have at least 20 different crystallin subunit polypeptides. However, despite this disadvantage, considerable progress has been made in studies of the control of crystallin synthesis. One special advantage offered by the vertebrate lens is that no lens fibres are lost during life and the constituent crystallins are preserved. Even the crystallins synthesized in foetal life still reside in the inner primary lens fibres. Therefore, by stripping away successive layers of lens fibre, it is possible to recover and analyse the complete ontogenic history of lens crystallin production. The successive layers of the lens are termed the nucleus which is the compacted inner region, the cortex of outer fibres and the equatorial zone which lies between the cortex and the peripheral epithelial region. The literature on crystallin ontogeny has been reviewed by Scheib (1965) and by Clayton (1970), but unfortunately there is very little data available on the comparative incidence of the crystallin subunits during ontogeny. The best analysis is that of Clayton (1970) with the so called "First Important Soluble Crystallin" or FISC in the chick. The incidence of subunits of this crystallin was followed from embryonic to adult life and the picture which emerges is that synthesis of these subunits is roughly progressive during development from stage 25 to hatching, with new bands appearing at stages 27 and 34.

A method which many investigators have used in assays for crystallins is that of fluorescent antisera raised against a particular crystallin preparation. Entire lenses may, therefore, be sectioned and challenged with the antisera and the localization of the antigen demonstrated by the colour distribution in the section. The method has, however, a number of pitfalls. First, there is the problem of cross reaction with other crystallins on the part of the fluorescent antisera. Second, since the sera are normally raised against a non-reduced crystallin preparation, a whole complex of antibodies is contained in the serum, some specific for one subunit and some for another. In addition, there is some contradiction in apparent findings which seems to be due to a masking effect on the antigenic determinants impaired by the high protein content of the central lens fibres. Antibodies have, however, been prepared against isolated β crystallin subunits and give a more reliable distribution pattern (Clayton, 1970). Interestingly, a differential distribution within the individual lens fibre is found, certain subunits apparently becoming concentrated at the periphery of some lens fibres. There are also reports of a non uniform distribution of crystallins in different cells in the same parts of the lens, especially in the primary fibres (McDevitt, 1968). Although these differences have been attributed to lack of synchrony amongst the primary fibre cells, it seems that differential loss or breakdown of the crystallins cannot easily be ruled out.

2. Ontogenic succession of crystallins

As we have already mentioned, it is relatively easy to determine the ontogenic succession of a particular crystallin by an assay on different parts of the adult lens. Such changes have been reported by several authors (see review by Waley, 1969). These changes have been detected chiefly by immunological means, either by comparing young and old lenses or by comparing epithelium, cortex and nucleus of an old lens. Crystallin synthesis is often apparent in the epithelial cells prior to the differentiation of these cells into secondary lens fibre cells.

The clearest conclusion on the subunit succession is the presence of an α crystallin subunit in the adult ox or chicken which is absent from the bovine or chick embryo. Since the subclasses of α crystallin appear to consist of the same subunits in different proportions, absence of one subunit in the embryo alters the composition of all of the α crystallins present. Another good example of ontogenic change of crystallin has been found in the lenses of the clawed toad (*Xenopus laevis*). In this amphibian, a γ crystallin is strongly represented in the tadpole lens fibres but is not synthesized in the adult (Campbell and Jones, 1968). It is not, unfortunately, absolutely clear from this work whether the γ crystallin is lost from the adult or persists only in the primary lens fibres.

3. The differential distribution of crystallins

One of the most intriguing questions in cellular genetics relates to the differential distribution of proteins. Are most proteins widely or universally distributed in cells but present in widely varying amounts (giving the illusion of a presence or absence distribution) or are many proteins the products only of specialized and highly differentiated cell lines? We have discussed this question under the headings of haemoglobin and insulin distribution also. Crystallins are normally taken to be proteins unique to the lens cells. Since powerful antisera can be raised against them, it is possible to expose other non-visual tissues to these antisera in a search for small amounts of crystallins in these other tissues. Cross reaction in antibody preparation and use must, of course, be always considered in the interpretation of the results of such experiments and in many of the examples these are particularly difficult to rule out.

The most frequent reports of reaction to anti-crystallin outside the lens itself comes from studies on brain tissue; in particular, rather strong reactions to anti-crystallin antibodies raised against *Xenopus* lens crystallin can be detected in *Xenopus* brain cells. Leakage of crystallins from the lens to the brain cells seems to have been ruled out as an explanation. Thus, although studies on crystallin do not prove the widespread incidence of crystallins in non visual tissues, they do give grounds for considering such a possibility. Clearly, more and better experiments are necessary to prove the point one way or the other.

4. Stability of messenger RNA

Since crystallin constitutes a major part of the complex of proteins synthesized in the cells of the lens, it is possible to follow their rates of synthesis by the provision of radioactively labelled amino acids and autoradiography of gel electrophoresced tissue extracts. Such studies, with and without the use of actinomycin D, can be expected to give some information on the life of the messenger RNA molecules controlling crystallin synthesis. Thus, Scott and Bell (1965) have found that, whereas in the developing embryonic lens actinomycin D caused a rapid drop in protein (and crystallin) synthesis, exposure of adult lenses to the drug had little or no effect on crystallin synthesis. A more detailed assessment by Clayton (1970) has shown that there is a varied response to Actinomycin D by different crystallins, α crystallin being considerably affected in the early epithelium but resistant later, while γ crystallins are always relatively insensitive. Actinomycin D does, of course, have other effects on cells besides inhibiting messenger RNA production, particularly its very striking effect on the synthesis of ribosomal RNA and an effect, at least in high concentrations, on cell membranes and permeability. For these reasons assessments of differential messenger RNA lifespan based on Actinomycin D experiments must be accepted with some caution.

Since potent antisera can be raised against the crystallins of the lens, an alternative approach to this problem has been the use of specific anti-crystallin antisera for the differential binding of polysomes involved in the synthesis of the particular antigen. Turnover of messenger RNA associated with these polysomes can then be determined. Such experiments broadly confirm the work with Actinomycin D in terms of progressive stabilization of crystallin mRNA with age and that such stabilization varies between mRNA molecules coding for different polypeptides. In particular, mRNA coding for crystallin appears to be more stable than that coding for lens enzymic proteins (Clayton *et al.*, 1972).

Lens crystallin messenger RNA has, in fact, been isolated and is apparently in the form of a functional ribonucleoprotein, the attached protein perhaps being involved in the differential life expectancy of these crystallin mRNAs (Williamson *et al.*, 1972).

The lens does, then, appear to be a tissue in which translational control of protein synthesis is achieved by controlling the life span of the mRNAs.

5. Other lens proteins

Besides the crystallins, other lens proteins also provide interesting subjects for study, particularly some of the enzymes. This is chiefly because of the lack of vascular supply to the lens, making an anaerobic metabolism essential. Thus, although mitochondria and oxidative enzymes occur in the epithelial cells, they are absent from the fibres, being replaced by a glycolytic metabolism. These changes are also paralleled by changes in the lactate dehydrogenase isozymes (LDH), from a predominance of LDH_5 to an abundance of LDH_1 (Papaconstantinou, 1967). This involves a dramatic shift from production or persistence of the LDH monomer A to monomer B since LDH_5 is A_4 and LDH_1 is B_4. Thus, changes in gene expression can be followed with some facility in the differentiation of the lens fibre.

6. Lens regeneration

Another interesting contribution of the vertebrate lens to studies on gene expression is made by its ability to differentiate anew from other eye tissues, if the original lens is removed. In tadpoles of *Xenopus laevis*, for example, it is reported that either retina, iris or cornea cells can produce lens fibres via a stage of dedifferentiation. Portions of cornea have been maintained in culture and are found to produce outgrowths of the lens tissue. The lens cells are identified both on grounds of morphology and immunological identification of lens-specific proteins. What is also of great interest is the ability displayed by pieces of the differentiated lens to suppress this corneal differentiation, suggesting that it is the presence of the lens which normally provides a feedback control on the cornea, preventing its development into the lens (Campbell and Jones, 1968). *In vitro* differentiation of lens fibre cells would, therefore, seem to be a most profitable subject for further investigation into the molecular signals, determining and controlling differentiation. Lens extracts of various kinds ought to display powers of control over the lens differentiation from the cornea.

7. Lens fibre differentiation *in vitro*

As we have already mentioned, lens fibres will differentiate *in vitro* from lens epithelial cells (Papaconstantinou, 1967 and Okada *et al.*, 1971). Attempts are now under way to utilize such an *in vitro* differentiating system to identify the controlling factors in the differentiation of the fibre cell and the synthesis of the crystallins. In such experiments, the epithelial cells, taken from embryonic or newborn rats, are found to lack γ crystallins (as determined by indirect immunofluorescent staining) and the initial outgrowing monolayers of epithelial cells in culture also lack γ crystallin. In some five days, however, differentiation to a fibre-like cell takes place and virtually all cells then stain positively for γ crystallin (Creighton and Trevithick, 1974). This work also reveals that cyclic AMP and caffeine have a strong potentiating influence on this differentiation, presumably by stimulating cell growth, and some exposed cells becoming positive for γ crystallin after 48 h rather than 5 days.

Although the influence of caffeine and cyclic AMP is probably fairly unspecific, it is encouraging to know that such a system as lens fibre differentiation and crystallin synthesis is being followed closely in tissue culture.

8. Comment

The lens provides a most unusual model for studies on an ontogenic succession of proteins. It has the advantages that (a) the cells with their specific constituents are preserved in an orderly sequence throughout life, making assay of the ontogenic sequence of crystallin synthesis relatively easy, (b) powerful antisera can be raised against lens crystallin and entire lens sections challenged with fluorescently tagged antibody and (c) the regeneration of the lens from the cornea and other tissues makes a useful *in vitro* system for studies on differentiation. Perhaps the greatest limitation to the usefulness of the system is the multiplicity of the crystallin subunits and the difficulties attendant on their correct separation.

The lens system can usefully be exploited further in terms of:

1. The factors involved in the inhibition of lens differentiation from cornea.
2. Studies on the switch from LDH monomer subunit A to B during lens fibre differentiation.
3. Controlled disaggregation of lenses so that single cell immunofluorescence can be carried out on isolated cells from selected areas of mature lenses. This would give a more clear cut result than the rather gross exposure of whole lens preparations.
4. The relationship between mRNA life span and ontogenetic sequence of proteins can perhaps be better studied with crystallin than any other protein. It might be possible to identify the crystallins, represented by mRNA preparations from lens tissue, by micro-injection of the mRNA into Xenopus oocytes. Such experiments, in combination with radioactive labelling of the message, should prove or disprove the thesis that mRNA for early crystallin is unstable and that for later crystallins more stable. Probes of cDNA made from crystallin mRNA would also help in such investigations.

E. Milk proteins

The mammary gland of the female mammal is an important experimental system. Its special relevance to the control of gene expression is, first, that its genetic commitment is relatively confined to the mass production of only a few proteins and, second, that its synthetic activity is very tightly geared to hormonal influence, thus presenting an excellent example of hormonally controlled gene expression.

Milk is directly the product of the mammary gland and, in detail, of the mammary epithelial cells. The epithelial cells are clustered in alveoli, each alveolar group being enclosed by a loose basket-like network of myoepithelial (presumably contractile) cells and surrounding an alveolar duct which drains the milk into the larger lactiform duct (Bloom and Fawcett, 1968; Saacke and Heald, 1974). Similar arrangements of cells are to be found in other epidermal glands such as sweat and salivary glands, to which the mammary gland is presumably related. Milk proteins are, therefore, the product of essentially one cell type, the mammary epithelial cell.

Actually, the true situation is slightly more complicated than that statement implies, since not all milk proteins are synthesized by the mammary epithelial cells, some being proteins which are found in the blood circulation and move into the milk through blood vessels permeating the gland. Studies by Barry (1961) and Askonas *et al.* (1955), using radioactively labelled amino acids, have demonstrated that such proteins as casein and B lactoglobulin were indeed synthesized by the gland cells. There is now reasonable evidence to indicate that the milk proteins synthesized by the mammary epithelial cells are α casein, β casein, α lactalbumin and β lactoglobulin. These proteins make up some 90–95% of the total milk protein by mass. Other proteins which appear in milk and are derived from the blood pool of circulating protein include serum albumin and immune β globulins. The origin of some milk proteins, such as the γ casein fraction, is still uncertain (see review by Larson and Jorgensen, 1974) and some may simply be degradation products of larger milk protein molecules (Gordon *et al.*, 1972). In addition to the major proteins, milk also contains a vast variety of minor proteins, including many enzymes (see Jenness, 1974).

Although the regulation of synthesis of lactoglubin and lactalbumin has been studied in the mammary gland, most work has centred on casein metabolism, chiefly because it is the most abundant of the milk proteins, accounting for about 80% of the total protein nitrogen in bovine milk. It is a family of substances which are actually phosphoproteins and are present in milk as calcium caseinogenate. The phosphate is believed to be linked to the β hydroxyl groups of the serine and threonine residues in casein. The casein may be separated, in most species, into α, β, γ and κ bands in order of decreasing electrophoretic mobility at alkaline pH, the α and β caseins being the most abundant. There is some question about whether or not the minor bands of γ and κ casein are actually phosphoprotein, since little phosphate is associated with these bands. Unfortunately, there is also evidence that there are actually more types of casein than this nomenclature would suggest, Wake and Baldwin (1961) isolating some twenty different bands in starch gel electrophoresis in urea solutions. For this reason, casein synthesis is analogous to crystallin synthesis, in constituting an attractive system for study but involving unfortunate complexity at the basic biochemical level. By contrast, α lactalbumin and β lactoglobulin are single well defined specific proteins, the latter being now recognized as a dimer of two identical chains, each of 162 amino acids (Kalan *et al.*, 1964/5).

Therefore, despite the abundance of casein in the milk of most mammals, there are good grounds for concentrating work on lactalbumin and lactoglobulin. All three proteins are, as far as is presently known, synthesized only by mammary epithelial cells and all have the advantage of being highly sensitive to hormonal control.

The methods of study of hormonal influence on milk protein synthesis have chiefly involved organ culture and both adult mammary gland slices and differentiating explanted embryonic glands have proved extremely useful (Turkington, 1968). Although there is a tendency for dispersed cell cultures of mammary gland epithelial cells to cease synthesis of milk proteins after some time (Larson, 1969), the cessation can be delayed by hormonal influence (Anderson and Larson, 1970). Subcellular fractions of the mammary gland have also been used for studies on milk protein production (Fraser *et al.*, 1959), but have so far proved less useful than the organ culture experiments.

1. Hormonal influences on mammary gland differentiation and synthesis

This topic has been reviewed by Turkington (1968) and involves studies on the influence of hormones on either total gland or gland explants, derived from early adolescent virgin female mice, on the one hand, or pregnant mice on the other. The main finding, in this work, is that differentiation of the gland to form alveoli and establish secretory cells requires the presence of insulin, hydrocortisone and prolactin. The requirement for hydrocortisone and prolactin is slightly unspecific, since aldosterone and some other adrenocortical hormones may replace the former and placental lactogen the latter. The requirement for insulin is, however, absolute. Although exposure of these organ cultures to insulin results in cell division, little or no differentiation of function is apparent while, in the presence of all three hormones, differentiation of alveolar structure is observed (Turkington, 1968).

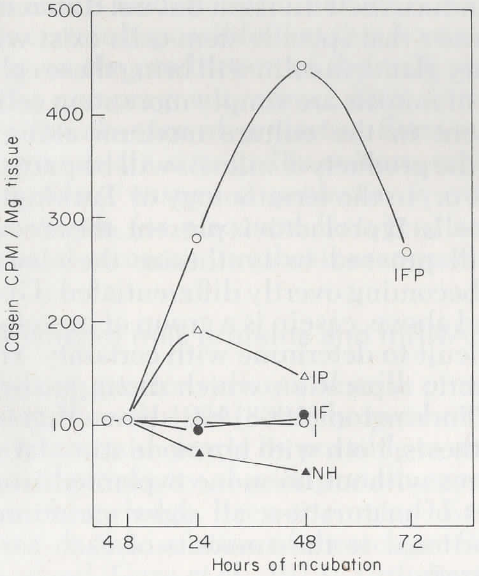


FIG. 6. Effects of various hormones on casein synthesis by explants of mouse mammary gland. The lines indicate the time course of incorporation of ^{32}P into casein by mid-pregnancy explants incubated in synthetic media and containing the hormones indicated. Each point represents incorporation during the preceding four h pulse-labelling period. I = insulin; F = hydrocortisone; P = prolactin; NH = no hormone addition (from Turkington, 1968).

Similar results are obtained when differentiated function is measured in terms of protein synthesis of casein, α lactalbumin and β lactoglobulin in explants of the mid-pregnant gland. As seen in Fig. 6, illustrating casein synthesis in explanted mid-pregancy mouse mammary gland, the three hormones involved display a synergistic effect on casein production. Similar but not identical curves have been obtained for both α lactalbumin and β lactoglobulin.

When the effects of the triple hormones are determined on the total protein synthesized by the gland explants, the hormonal influence is found to be highly selective. Insulin alone will maximally affect the total epithelial cell synthesis of non-milk proteins (Lockwood *et al.*, 1966), no doubt largely due to cell proliferation. Likewise, synthesis of non-casein phosphoprotein, in the presence of the three hormones in combination, is 50% greater than without the hormones, but in the case of the casein phosphoprotein it is 300% greater.

Attempts have been made to distinguish the particular effects of the three hormones concerned. As we have already mentioned, insulin alone induces cell division but not differentiation. If one, therefore, assumes that specific stem cells exist within the uninduced mammary gland, insulin will bring these cells into mitosis, but the results of mitosis are simply more stem cells. If hydrocortisone is present in the culture medium along with insulin, however, then the products of mitosis will be partly differentiated epithelial cells or, in the terminology of Turkington, "covertly" differentiated cells. If prolactin is present, these covertly differentiated cells will proceed to synthesize the characteristic milk proteins, thus becoming overtly differentiated (Grobstein, 1964).

As mentioned above, casein is a group of proteins and the exact number is difficult to determine with certainty. However, using an electrophoretic separation which distinguished six separate casein bands, Turkington (1968) has shown that increases in the net casein synthesis, both with hormone stimulation *in vitro* and in organ cultures without hormone explanted from the gland at different stages of maturation, all show variations in synthesis roughly proportional to the amounts of each casein band normally synthesized.

2. The insulin effect and cell division

The mammary gland system demonstrates an interesting relationship between cell division and synthesis of specific proteins. For example the triple hormones show a marked augmentation of synthesis of milk proteins by explants of mid-pregnancy mammary gland. This augmentation can be totally abolished by the inclusion of colchicine in the culture fluid (Stockdale and Topper, 1966). On the other hand colchicine does not apparently interfere with the milk protein production of the post-mitototic differentiated cells (Turkington *et al.*, 1967).

These and other results taken together indicate that casein synthesizing cells are produced only by insulin-mediated cell proliferation, occurring in an appropriate hormonal environment, as provided by prolactin and hydrocostisone. Pre-existing differentiated cells cannot be transformed, in these incubations, into milk-producing progeny cells; only the daughter cells of undifferentiated stem cells can be converted into cells synthesizing milk protein.

3. The specific effect of prolactin

The post mitotic induction of casein synthesis (and indeed of γ lactalbumin and B lactoglobulin) is highly dependent on prolactin and it is certainly possible that this hormone is acting in a very direct way on the production of the milk proteins. Thus, in the gland of the non pregnant animal, little or no casein will be synthesized in the absence of prolactin. Even when explants of mid-pregnant glands are arranged in culture, rapid loss of casein synthesizing activity ensues if prolactin is omitted from the medium. If prolactin (or placental lactogen) are subsequently added, however, then casein synthesis reappears.

4. Genetic variants of milk proteins and mRNA isolation

Since a number of genetic variants of milk proteins are known (Thompson and Farrell, 1974), chiefly involving alterations in one amino acid, it would clearly be possible to undertake studies on the possible multiplicity of milk protein genes. Such studies have not, to my knowledge, been attempted. Experiments have been carried out on the isolation of messenger RNA from lactating mammary gland (Gaze *et al.*, 1973). Unfortunately, the extent of commitment of the mammary epithelial cell to the production of any one protein is not very great, but at least some of the 15S RNA isolated from the ribosomes of ewe mammary glands by Gaze *et al.* (1973) did direct some casein synthesis when employed in a cell free system.

5. Linkage of the milk protein genes

It has been known for some years that some chemically related proteins are the products of genes which are closely linked. Such an arrangement might be thought to favour the coordinate control and expression of such genes. Examples were cited in the chapter in this book on fungal systems (Section 4D) and other examples are possibly the egg-white proteins and β and γ chain haemoglobins (Boyer *et al.*, 1963). Of course some of these examples may simply betray recent evolutionary divergence with complete absence of coordinate control. In any event, there is good evidence of close linkage of the genes coding for α, β and κ caseins, the links between the former two genes being extremely close (King *et al.*, 1965; Grosclaude *et al.*, 1965).

6. Comment

Since the extent of the commitment of the mammary epithelial cell to the production of one or two protein species is less than is the case with reticulocytes or cocoonase synthesizing cells in insects, for example, the mammary gland system is certainly not the best choice for specific messenger RNA isolation or for studies on control of expression of one or two genes. But the interesting control of milk protein synthesis exercised by insulin, progesterone and prolactin, both during early gland differentiation and in the synthetic activity of the maturing gland, is of very great value as an experimental system. The insulin modulated cell division, which acts as a prerequisite for the later progesterone and prolactin induction of specific protein synthesis, should not, however, be used to argue that cell division and differentiated function are always separate acts of commitment with a fixed order relative to one another. Haemoglobin synthesis often or always begins before the terminal mitosis of the differentiating erythroid cell—see discussion in Section 3C. But the important relationship between cell division and genetic commitment is certainly worth noting, since it is an aspect of the commitment of many other cellular systems.

F. Fibroin

Fibroin is the main protein of silk, and is a fibrous protein of molecular weight about 170 000 (Tashiro and Otsuki, 1970) consisting primarily of alternating glycine and alanine residues with lesser amounts of serine and very small amounts of other amino acids (Lucas and Rudall, 1968). Silks are produced by very many insect and Arachnid (spider) species and the silks of differing species have widely variant amino acid compositions. Indeed, some species, particularly spiders, are able to manufacture more than one type of silk since they possess multiple silk glands, each gland producing a product with particular characteristics of strength or stickiness. Actually, the silk threads spun by many anthropods consist of a core of fibroin covered in a sheath of a different protein, sericin. The sericin acts like hard varnish on the silk, making the threads strong and enabling them to harden together into a web or cocoon. The cocoonase enzyme, discussed in Section 3G of this book, used by some silk moths to soften the walls of their cocoon, is in fact an endopeptidase highly specific for sericin (Kafatos and Williams, 1964).

Fibroin is of interest to us in the context of this book because, like cocoonase, it is a protein made in very large amounts by a few highly specialized cells. Its storage and extrusion as an extracellular product in silk make it easy to recover and purify, while the extreme dedication of the silk glands to its manufacture has facilitated the isolation of fibroin messenger RNA.

1. Polyploidy of the silk glands

The cells of insect silk glands are enormous and extremely polyploid. Those which have been closely studied are of the common silk moth *Bombyx mori.* A single cell within the silk gland of this species contains as much as 0·2 μg of DNA of which 0·0022% codes for fibroin mRNA (Tashiro *et al.*, 1968). The amount of fibroin synthesized by such a cell amounts to 300 μg, mainly during the last four days of the fifth instar of larval development. Indeed until the end of the fourth instar, the silk glands remain small but during the fifth instar the glands increase very greatly in size. This size increase is entirely due to enlargement of the individual cells, since the total number of cells in these silk glands remains constant throughout postembryonic development (discussed in Tashiro, 1968). It also appears that the dramatic increase in cell size precedes the intense fibroin production by the cells. Cell size increases by a factor of about 500 000, giving a DNA content in these cells of about 10^6 times the haploid amount (Gage, 1974).

2. Transcriptional and translational control in fibroin synthesis

It would appear that the massive and rapid production of one protein expected of the silkworm silk gland cells would ideally be achieved by specific amplification of the fibroin gene. Gene amplification—production of many additional copies of a particular gene for a temporary increase in transcriptional production—is well known to occur in the amphibian oocyte with the genes for ribosomal RNA (see Section 6A). There has been widespread speculation amongst biologists that amplification could well be used as a mechanism for localized production of large amounts of one or two proteins. It is, therefore, particularly impressive to notice that, in the case of fibroin where production of one protein is possibly at its extreme (Suzuki *et al.*, 1972), no evidence for specific gene amplification exists. Instead, the entire genome is replicated to yield one million times the DNA content of the silkworm sperm, involving an increase from about $0{\cdot}4 \times 10^{-12}$ gm of DNA to $0{\cdot}2 \times 10^{-6}$ gm of DNA in the diploid cells. Each cell, therefore, comes to possess about 10^6 copies of the fibroin gene and their transcription and translation results in the production of about 10^{15} fibroin molecules. For this production, about 10^{10} fibroin mRNA molecules are made per cell and, since these molecules appear to be very stable, this implies that each fibroin gene produces about 10^4 messenger RNA molecules, each of which can be translated into 10^5 protein molecules in five days. Such a production involves a programme of protein synthesis which polymerizes amino acids at the rate of 15/s/ribosome. All of these calculations are derived from the papers of Suzuki *et al.* (1972) and Suzuki and Suzuki (1974).

Suzuki *et al.* (1972) have also concluded that the efficiency of transcription of the fibroin genes is no greater than that of the genes for ribosomal RNA, if the accumulated products can be used for the comparison. Thus, 85% of the total RNA content of the mature silk gland cell is ribosomal and 1% fibroin messenger and the ribosomal genes are about 80 times more numerous than those for fibroin—the ribosomal genes account for about 0·17% of the genome in *Bombyx*.

Perhaps the most impressive aspect of fibroin production is the efficiency with which the fibroin mRNA is conserved and translated. The fibroin mRNA appears to be very stable during the period of its production and use, but is very rapidly degraded when the period of moulting ensues (Suzuki and Suzuki, 1974). It would be most encouraging to find that there was a substantial poly A sequence attached to fibroin messenger RNA, in view of its suggested influence on messenger RNA life span, but in fact Suzuki and Brown (1972) seem to have ruled out the possibility of more than 10 poly (A) consecutive sequences in the molecule.

Although there is no evidence for selective amplification of the fibroin genes, it should be stressed that the precise number of fibroin genes in the silkworm genome is not known, but only inferred from hybridization studies. The number is almost certainly between one and three copies per haploid genome (Suzuki *et al.*, 1972; Lizardi and Brown, 1973). The latter authors conclude that the fibroin gene should consist of $11\text{–}12 \times 10^6$ daltons of double stranded DNA, which ties up with the figure of 0·002% of the *Bombyx mori* genome mentioned earlier, which is actually the hybridization saturation value for labelled fibroin mRNA binding to native *Bombyx* DNA.

The translational commitment of the fifth instar silk gland cell to fibroin production is obviously considerable. If 10^{10} fibroin mRNA molecules are being translated at that time and 50 ribosomes are utilized in each fibroin polysome, then 5×10^{11} ribosomes are entirely committed to fibroin synthesis in these cells. If the number of ribosomes per cell increases proportionately with increased cell size and ploidy, then 1×10^6 fibroin-committed ribosomes would exist proportionately in a diploid cell of *Bombyx*. Actually that may not be a large proportion of the total population, since an average diploid cell might be expected to contain perhaps a few hundred million ribosomes (working from the data of Maclean (1965) that a yeast cell contains $0{\cdot}5 \times 10^6$). If these calculations are at all near the mark, they imply that the translational commitment to fibroin synthesis, though massive, may yet only account for a smallish proportion of the entire ribosome population.

One of the most intriguing observations made on the fibroin synthesis system is that the fibroin messenger RNA production, measured as a percentage of the total cellular RNA, is relatively independent of cellular morphology and even ploidy during the third, fourth and fifth instars of silkworm growth. In other words, since most of the cellular RNA is ribosomal, there is little change in the ratio of fibroin mRNA to rRNA (Suzuki and Suzuki, 1974; Gage, 1971). Such a parallel synthesis suggests a lack of fine modulation and a gene control mechanism which simply permits continuous maximum transcription to proceed for these genes in these cells.

3. Isolation of fibroin messenger RNA

Fibroin mRNA is, to date, the only eukaryotic mRNA which has been isolated in sufficient bulk and purity to permit its chemical characterization (Suzuki and Suzuki, 1974; Suzuki and Brown, 1972). Its isolation and characterization have been greatly facilitated both by the extreme commitment to fibroin synthesis which characterizes the silk gland cells and by the unusual and rather regular amino acid sequence of the fibroin itself. This implies that the fibroin RNA will have a relatively simple sequence and unusual base composition, so rendering its separation as an RNA satellite feasible in the ultracentifuge.

The extraction procedure involves removal of the posterior silk glands from fifth instar larvae, previously labelled by injection of $^{32}PO_4$ during the first part of the fifth instar. Total RNA is extracted from these glands (Suzuki and Brown, 1972) and up to 130 mg aliquots applied to Bio–Gel column for fractionation and elution at 25°C with a Tris, EDTA, sodium dodecyl sulphate buffer. The fibroin mRNA can be eluted as a discrete peak well separated from the ribosomal RNA. Its identity as fibroin mRNA depends on many criteria. First, its high molecular weight is suggestive, since fibroin mRNA might be expected to have a weight of about $2{\cdot}3 \times 10^6$ daltons. Second, the known structure of the repetitious polypeptides which make up about 75% of *Bombyx* fibroin predicts a minimum G + C content of the total mRNA of 57%, which is higher than any other cellular RNA component except 4s RNA. Moreover, the G content of the mRNA should itself be about 40%. The isolated mRNA has, in fact, a G + C content of 59% and a G content of 40% (Suzuki and Brown, 1972). By following the decay of the label in this mRNA, it has also been possible to show a long half life of many hours and more probably a few days (Suzuki and Brown, 1972) and this again is as expected for the fibroin messenger RNA.

4. Comment

The system of fibroin synthesis by the insect silk gland comes close to the ideal for studies on the control of gene expression. The messenger RNA can be isolated in bulk with relative ease, the DNA containing the fibroin gene can be concentrated by dint of the unusual base composition of the gene and the commitment to fibroin synthesis is short term in the life of the insect and very localized in the tissues of the organism. These reasons have all contributed to the success of the experiments on determining the number of copies of the fibroin gene in the genome—less than four and probably one. No other eukaryotic gene has been so narrowly identified. What more can we expect this outstanding research system to produce?

There are perhaps two immediate problems whose resolution seems possible in this system. One is a study of the factors which determine mRNA life span. Fibroin mRNA is long lived but, when the time comes, apparently degraded rather quickly. Is this pattern of survival built in to the mRNA or is it to be understood in terms of changing cellular environment? Gurdon's injection of globin mRNA in *Xenopus* eggs and embryos (see Section 4E) suggests, but does not prove, the latter explanation. The other problem, to which reference has often been made in this book, is the efficiency of transcriptional repression in tissues not normally expressing a gene. The unusual characteristics of fibroin mRNA, and even of fibroin itself, should make it possible to construct sensitive probes for the detection of low concentrations of fibroin, or fibroin mRNA, in silkworm tissues other than the silk glands. The complementary approach, that of initiating fibroin mRNA production and or fibroin synthesis in tissues not normally producing silk, would also become feasible if sufficiently sensitive assays for these products were available.

It would also be most useful to know what proportion of the silk gland ribosomes are involved in fibroin assembly in the fifth instar larva. Since it is essentially a product for export, such observations should also shed light on the old suggestions, never altogether proved or disproved, that protein for export is made only on membrane bound ribosomes, while the free ribosomes are chiefly or exclusively involved in the synthesis of intracellular enzymes.

G. Cocoonase

As is well known, the silk moths are characterized by the silken cocoon spun by the larva as a protection for the resting pupal stage. The cocoon does, however, confront the hatching moth with an escape problem, since most cocoons are entire and without an exit. Some species do make a trap door during cocoon construction but, for most, breakdown of part of the cocoon is necessary for hatching of the adult. In a few cases, the weave of the cocoon is loose and flimsy and the moth is able to push its way through but, most frequently, the structure hardens shortly after spinning into a tough and rather thick walled cell. Silk moths which construct cocoons of this type escape from them by digesting the silk with a specific cocoonase enzyme secreted from the paired galea or mouth-parts of the moth. This cocoonase enzyme and the cells of the galea which synthesize it, constitute an interesting if minor research system, in which problems of differentiation and gene expression may be studied. A recent review by Kafatos (1972) provides an excellent discussion of this field.

The cocoonase enzyme is synthesized by only about 40 000 polyploid cells in all, the cells being located on the lateral surfaces of the galea. Moreover, late in adult development, the enzyme is extruded to the surface of the mouthparts where it collects and dries into a semicrystalline mass which may be picked off by forceps. Each moth may yield up to 0·1 mg of the solid, rendering analysis of this protein extremely simple. The semi crystalline mass is apparently almost pure enzyme (Kafatos, 1972). During hatching, the adult moth secretes an isotonic solution of $KHCO_3$ from a neighbouring gland, the enzyme readily dissolving in this solution and proceeding to digest the silken fibres of the cocoon.

1. Chemical characteristics of the protein

Because of its relative purity when collected and the comparatively large amounts recovered from each insect, the protein has been well characterized. It is a trypsin-like protein of about 24 000 molecular weight which, when first synthesized, is in the form of an inactive zymogen. Activation occurs soon after synthesis and all of the material collected from the galea is invariably fully active. Although early activation may depend on other proteolytic enzymes such as moulting fluid proteases, the process soon becomes autocatalytic, as in the activation of trypsinogen to trypsin. The zymogen molecule of cocoonase has a molecular weight of approximately 30 000, so activation involves loss of a substantial portion of the polypeptide chain.

2. Synthesis of the protein

According to Kafatos (1972), cocoonase zymogen synthesis proceeds at an accelerating rate between day 9 and day 14 of adult development within the cocoon, up to a maximum synthetic rate of $1{\cdot}5 \times 10^{10}$ molecules per zymogen-cell per day which is some 60% of the total protein synthesized by the cells of the galea at that time. By any standards that represents very impressive commitment to the manufacture of a single protein on the part of a very specialized group of cells.

The period of cocoonase zymogen synthesis can be subdivided into different stages or phases, as can the protein synthesis of differentiating pancreas cells (Rutter *et al.*, 1968b). In the first phase, the rate of enzyme synthesis is very low; this stage has been called protodifferentiation by Rutter *et al.* (1968b). During the second phase, on day 8 or 9 of adult moth development, the rate of synthesis rapidly increases from a low level and climbs from 1–70% of all protein made in the zymogen cells six days later. Then stage three ensues, when the rate of zymogen synthesis apparently decreases rapidly.

A number of interesting points about gene expression and the control of protein synthesis can be raised, at this stage, in relation to cocoonase. First, prior to the protodifferentiation phase, is any cocoonase synthesized at all? Second, in this system, what factors determine the rapid synthesis and accumulation of this protein? Third, during the final phase, are we witnessing actual regradation of enzyme or of its messenger RNA, or is it simply a dilution of these proteins and these differentiated cells by other proteins or other cells?

I have remarked elsewhere in this book about the lack of information on the amounts of protein, present in cells, which are taken to be baseline amounts and also about the possible widespread occurrence of these proteins at low concentration in other tissues. The question is not at present clearly answerable for cocoonase, since even the predifferentiated cells of the galea demonstrate a low level of cocoonase activity. The decision to differentiate zymogen cells in the galea is actually made some days before the onset of stage 1 anyway. This can be shown by modulating the hormones which control the differentiation. Ecdysone activity alone leads to the epidermal cell differentiation into zymogen producing cells, but if the insect juvenile hormone is also present, zymogen cell differentiation is inhibited. It has been found that juvenile hormone is no longer effective in inhibiting the differentiation after day 3 or 4 of adult development, however (Williams, 1968). Some days later ecdysone itself becomes a dispensable hormone to the ongoing differentiation process. Clearly, both hormones act as trigger factors for internally coded programmes of development. Some cocoonase is apparently present in the predifferentiated galea cells, therefore, but it is not known whether or not it is present in other unrelated tissues of the larva, pupa or adult.

Moving to the question about the factors affecting the transition from phase one to phase two and the rapidly increasing rate of cocoonase synthesis and cellular dedication to its synthesis, the information available is more detailed and precise. The first point to make is to emphasize the statement made in the previous paragraph, that the phase is internally programmed. Altering external hormonal levels at this stage of differentiation has no effect. Moreover, extraction of the zymogen cells on day 7 or 8 of adult development, followed by their culture *in vitro*, has little or no effect on the progression of phase 2 and the rapid increase in the rate of cocoonase production (Kanbysellis and Williams, 1971). But we must ask how one protein comes to be produced in such large quantities. Is it at the expense of the synthesis of other proteins, or is it in addition to the continued normal levels of synthesis? The answer from the available data, although somewhat indefinite on this point, seems to be the former. What is most clearly demonstrable from the data is that the increasing production of cocoonase is due to an escalation in cocoonase mRNA synthesis, coupled with a very long life span for the cocoonase mRNA—at least a half life of 24 h. Ths mRNA stability is specific to the cocoonase messenger as determined by the effects of high levels of actinomycin D and is apparent even prior to the onset of the second stage of zymogen cell differentiation. Quantitative interpretation of the absolute rate of an RNA production is complicated by the fact that DNA synthesis occurs actively during phase 1. But in any event, during phase two, protein synthesis appears to be directly proportional to mRNA content (the limiting effect of mRNA amount was also clear in Gurdon's mRNA injection experiments discussed in Section 4E).

Assuming that only one gene copy for cocoonase exists and that no gene amplification occurs, we must conclude that by activation or derepression, the level of cocoonase mRNA increases during protodifferentiation and stage 2. Combining this with a specially long half life for the cocoonase messenger, this will result in rapid accumulation of cocoonase mRNA and a rapid increase in the rate of synthesis of the enzyme.

It should be made clear that the transcriptional activation of the gene is purely speculative and deserves close study, but perhaps depends on the ability to identify a specific mRNA in small amounts, at present an impossible assignment.

Unfortunately, little is known about the run down in the synthetic rate characteristic of stage three. This is a pity, since some answerable questions seem to appear in this context, e.g. does cocoonase mRNA production cease; is there evidence for specific breakdown of cocoonase mRNA; is the enzyme itself degraded, or does the fall-off in the zymogen synthesis simply reflect a general decline in the metabolism of these differentiated cells.

3. Comment

The differentiating system of cocoonase zymogen-producing cells in some silkworms deserves to be better known. It has a number of very great advantages as a research system. By far the greatest is the astonishing commitment of these cells to the production of a single polypeptide. The second advantage is the ability to recover and characterize this protein after its extrusion from the galea, so making it easy to raise a comparatively pure antibody against it. The role of ecdysone and juvenile hormones in affecting the differentiation of the zymogen cells also provides a fairly simple model of hormone action.

Some questions seem to be open to solution in this system more than in others. They are:

1. Is cocoonase present in other tissues besides the galea of the developing moth and, if so, in what quantities? Challenge of tissues and tissue extracts with fluorescent antisera raised against the cocoonase surely provides a means of answering this important question.
2. Antisera might also present a means of identifying mRNA messenger complexes involved in the synthesis of this protein, thus providing a means of asking whether, when the cocoonase accounts for 70% of all protein made, the cocoonase committed polysomes are also 70% of the ribosomal population.
3. The rapid fall in cocoonase synthesis after day 15 of adult development also deserves study as a possible case of control by selective degradation of mRNA or protein or both.

H. Vertebrate egg proteins

In many vertebrates the production of egg protein makes a considerable demand on the synthetic resources of the organism, either because the eggs are relatively large, as in birds, or extremely numerous, as they are in fish and amphibians. The proteins are not synthesized in the egg but are made elsewhere, in the liver or oviduct tissue for example, and transported to the developing oocyte for storage. The proteins of the yolk and white of vertebrate eggs are very different and each are proving, in their own way, interesting systems for the study of the regulation of protein synthesis. We will discuss the two groups of proteins separately.

1. Egg yolk proteins

Production of these proteins occurs exclusively in the liver, and is normally a feature only of the female liver under the influence of oestrogen. If males are artificially treated with this hormone, however, they too will elaborate large amounts of egg yolk protein in the liver and export it from there to the circulation. This topic has been recently reviewed by Clemens (1974). The main yolk proteins are the lipovitellins, phosvitin and livetin, of which the first two form the bulk of the protein present. Multiple forms of these proteins are present in at least some species. Lipovitellin and phosvitin are phosphoproteins, the former also being conjugated with lipid. The former is apparently a dimer, and can be dissociated into two subunits of about 200 000 daltons each at alkaline pH (Wallace, 1965). Phosvitin is a smaller protein of about 40 000 and seems to exist as a monomer in physiological conditions.

One of the most interesting aspects of the production of these egg yolk proteins is that in studies of both the chicken (Wallace, 1970) and *Xenopus* (Clemens, 1974), there is good evidence to suggest that a single precursor molecule exists as the primary product of the liver cells, yielding phosvitin and the lipovitellin subunits only after its cleavage in the growing oocyte. The precursor molecule has been termed vitellogenin. Evidence that the liver is the main or sole site of vittelogenin production is strong and liver explants in culture have been shown to make and export this protein (Munday *et al.*, 1968). We will now discuss aspects of production and processing of this interesting protein which are of special relevance to our main topic.

(a) *Interaction of oestrogen with the liver*

Since the synthesis and secretion of the yolk proteins by amphibian and avian liver are oestrogen regulated, it is appropriate to discuss here the action of these hormones on the liver. As steroid hormones (see discussion in Section 7C) we might expect oestrogen to enter the appropriate liver cells and to be translocated by a carrier protein to some site of primary activity. Although there is evidence for the existence of such a receptor/carrier protein in the livers of laying hens (Arias and Warren, 1971) and even of induced synthesis of receptor protein by oestradiol (Mester and Baulieu, 1972)—the receptors isolated by these two groups of workers do not seem to be necessarily the same protein—there is unfortunately no evidence yet of the precise destination of the oestrogen/receptor complex other than the location of the nucleus. Whether or not the destination is indeed the nuclear chromatin, or even a particular sequence of the nuclear DNA, remains entirely speculative. Similar studies on the role of estradiol on the synthesis of phosvitin in chicken livers have been reported by Talwar *et al.* (1974).

(b) *Induction of yolk protein production*

Although the production of yolk protein by the liver is clearly induced by oestrogens *in vivo*, it has not yet proved possible to accomplish induction *in vitro* (Clemens *et al.*, 1974). The *in vivo* system is, however, dramatic in its response. Within a few hours of oestrogen injection, substantial plasma levels of egg vitellogenin can be detected both in the chicken and in *Xenopus*. Injection of the hormone into the male animal constitutes a particularly effective experimental system as no endogenous hormonal production is evident. When liver slices are prepared from *Xenopus* previously induced by oestrogen injection, up to 99% of the protein secreted by the tissue is vitellogenin (Clemens, 1974). Indeed the blood of such injected male frogs changes colour following injection and the animals finally die, apparently from the inability to rid the blood of the circulating protein (Tata, personal communication). Details of the biochemical events, following induction together with an interesting electron microscope study of the changes in cell ultrastructure during vitellinogen synthesis, will be found in Clemens (1974) and in earlier work cited by Clemens.

(c) *Uptake of vitellogenin by the ovary*

In the normal female bird or amphibian, the yolk protein which is made in and released from the liver in response to oestrogen is, in turn, withdrawn from circulation by the ovarian cells and transported into the developing eggs. In *Xenopus*, the plasma vitellinogin in the induced female has a circulatory half-life of two days; the half-life in oestrogen treated males is 40 days (Wallace and Jared, 1968). These authors have also shown that ovariectomy of the induced female gives a vitellinogen half life similar to that in the injected male, demonstrating that the ovary is responsible for the short half-life in the normal female. Radioactive labelling has also shown a precursor product relationship between the protein in the serum and in the ovary. The uptake and utilization of vitellinogen by the ovary involves pinocytotic uptake of a highly selective nature (Wallace and Jahred, 1969), but this is a topic somewhat marginal to our present subject. The processing of the vitellinogen within the oocyte to yield the egg yolk proteins lipovitellin and phosvitin has already been mentioned above. It is certainly a most interesting example of post-translational control of gene expression. The yolk proteins formed from the vitellinogen precursor are then utilized in the formation of yolk platelets within the egg. A platelet structure comprising two phosvitin molecules conjugated (non-covalently) with a lipovitellin dimer has been proposed by Wallace *et al.* (1972), although these authors suggest that the platelet structure may be slightly more complex than this. In any event, the final phenotypic expression of the egg yolk protein genes involves a structuring of the products of translation far distant from the site of their original production.

2. Egg white proteins

In the eggs of birds and some other vertebrate groups, the provision of a bulky egg white is accomplished during egg maturation and the egg white proteins are synthesized in the mucosal cells of the oviduct. A number of distinct proteins are to be found as constituents of egg white, e.g. ovalbumin, conalbumin, ovomucoid, lysozyme, and avidin. The first four account for about 90% of the total protein content of the egg white, avidin being perhaps the best known of the minor egg white proteins.

(a) *Sites of synthesis*

The chicken oviduct magnum can be subdivided into four classes of cells: (a) epithelial goblet cells, (b) ciliated cells, (c) tubular gland cells and (d) interstitial cells, including fibroblasts. An early study of the distribution of ovalbumin and avidin within the oviduct of the oestrogen treated chick suggested that ovalbumin synthesis occurs in the tubular gland cells and avidin in the goblet cells (Kohler *et al.*, 1968), but more recently Palmiter and Gutman (1972) carried out a localization study using fluorescent antibody raised against ovalbumin, conalbumin, ovomucoid and lysozyme. They concluded that all these proteins were made in the tubular gland cell, not in any of the other cell types, and also that, almost certainly, any one tubular gland cell made all four proteins.

(b) *Regulation of egg white protein synthesis by steroids*

One of the attractive features of egg white protein synthesis is that, like the egg yolk protein production, it is tightly controlled by steroids. Indeed, the effects of oestrogen on the uterus and oviduct have been studied very intensively from the point of view of the site of action of the hormone (Means and O'Malley, 1972). As in the case of oestrogen induction of egg yolk synthesis in liver, there is good evidence for binding of hormone to receptor protein and subsequent transport to the nucleus, but little evidence about the precise binding site within the nucleus. The response to induction in terms of synthesis of ribosomal and other species of RNA is reviewed by Means and O'Malley (1972) and has been further studied by Rhoads *et al.* (1973) and Palmiter (1973) from the point of view of the production and use of ovalbumin mRNA. Ovalbumin mRNA has been partially purified by these authors and the speed and fidelity of its translation studied in a cell-free protein synthesizing system. Relatively pure preparations of ovalbumin mRNA have been prepared by Cox *et al.* (1974).

Palmiter (1972) has also studied the kinetics of steroid hormone induction of the different egg white proteins by the chick oviduct, looking for evidence of co-ordinate or non-coordinate synthesis. Despite the fact that the four proteins surveyed are known to be made in the same cell (Palmiter and Gutman, 1972), Palmiter's evidence points strongly towards non-coordinate synthesis. In particular, the ratios of conalbumin and ovalbumin changed considerably during various phases of oestrogen stimulation. Different hormonal combinations also demonstrate non-coordinate responses, since the synthesis of ovomucoid and conalbumin increased relative to ovalbumin when either testosterone or progesterone was applied with the oestrogen. Some of the kinetic differences are most satisfactorily explained by assuming that the relevant mRNA molecules have different half-lives, but even this assumption does not permit any interpretation analogous to a bacterial operon or regulon.

As with the induction of egg yolk protein by oestrogen, the oviduct cells become involved in a relatively massive synthesis of tRNA and rRNA and a major ultrastructural escalation of endoplasmic reticulum, all as an accompaniment to the enhanced egg-white protein following hormonal induction (Palmiter, 1972).

The papers of Cox and his collaborators (Cox, 1973; Cox *et al.*, 1972, 1974) provide an interesting account of experiments on the fidelity of transcription of chick oviduct chromatin by different polymerase enzymes and on the amounts of ovalbumin mRNA present in hen oviduct cells before and after hormone induction.

Cox *et al.* (1974) have exploited their relatively pure preparations of ovalbumin mRNA to synthesize a radioactive complimentary DNA probe, using viral reverse transcriptase enzyme. Using this probe, they calculate that whereas the fully induced oviduct tubular gland cell possesses approximately 36 000 ovalbumin mRNA molecules, the uninduced cell contains approximately one such molecule. Not only is this impressive evidence of hormonal induction, but it comes extremely close to being sound and concrete evidence for complete transcriptional inactivity prior to induction, thus supporting a model of induction suggestive of specific gene derepression (or induction) rather than modulation of the amount of RNA synthesized (rate control). This experimental approach is of the greatest significance to theories of gene regulation and expression, particularly since the ovalbumin system is sufficiently explored to indicate that only two copies of the ovalbumin gene are present in the diploid oviduct cell, that gene reiteration does not occur after steroid treatment and that the ovalbumin mRNA has a half life of at least 24 h in the presence of oestrogen (discussed by Cox *et al.*, 1974).

3. Comment

The egg proteins of amphibians and birds, both yolk and white, provide excellent examples of a number of different specialized aspects of gene expression. There is tight regulation by steroid hormones; synthesis that occurs in an organ is very distant from the egg and, therefore, the product is exported from the productive cell; multiple proteins are in each group, permitting studies on co-ordinate synthesis; and uptake and processing of the proteins by the ovary or egg is necessary to form the storage proteins of the egg. The main conclusions to date are that (a) the precise chromosomal site of the steroidal activity has not been determined and (b) the transcriptional response of the target cells does not suggest a genetic unit with coordinate control, i.e. an operon or regulon.

The use by Cox *et al.* (1974) of the chicken oviduct chromatin system and of an ovalbumin cDNA probe is an excellent example of the application of recent technology to one of the really basic questions of the control of gene activity. In this regard, our understanding of eukaryotic control is moving much closer to that of the prokaryotes. In particular, this experimental approach permits direct measurements of gene activity rather than the very indirect inference which commonly follows from studies on protein synthesis and activity.

4. Experimental Systems of Differential Gene Function in Eukaryotes—Systems of Limited Complexity

A. Pancreas 104
B. Enzyme synthesis during the yeast cell cycle 111
C. Muscle 117
D. *Aspergillus* and *Neurospora* 130
E. Nuclear transplantation and microinjection 136
F. Cartilage 151
G. Liver 158
H. DNA-associated proteins 165

A. Pancreas

Studies on the control of gene expression in the pancreas involve more than one differentiated cell type. There are two distinct parts of the pancreas. The exocrine portion of the gland secretes a variety of enzymes into the digestive system via pancreatic ducts, the enzymes being synthesized in the acinar cells of the exocrine pancreas tissue. These enzymes include amylase, trypsinogen, lipase and other degradative digestive enzymes. Some of these proteins are initially produced as zymogens, or inactive forms, being later trimmed down by the breaking of some of the peptide bonds to yield the active enzyme.

The other part of this organ is the endocrine region, composed of groups of cells termed the Islets of Langerhans. Within these cellular islands are two types of cells, the A cells which make the protein glucagon and the B cells making insulin. The A cells lie in the periphery of the islets, while the smaller B cells lie mainly in the centre of the islets. They may be distinguished from one another, not only by size and general location but also by the morphological differences in the granules which they contain. This difference is particularly marked in the electron microscope, making possible an ultrastructural study of pancreas development and differentiation (Picket *et al.*, 1972). In some lower vertebrates the endocrine and exocrine portions of the pancreas are quite distinct and separate, rather than being fused into one organ as in mammals.

Both portions of pancreas have attracted the attention of cell biologists in their quest for populations of cells committed to the production of specific proteins. We will discuss the two portions

of the gland separately, although many authors and experimentalists have taken them together.

1. Exocrine pancreas

The particular attraction of the acinar cells of the exocrine pancreas is that these cells synthesize and secrete a variety of different enzymes as mentioned above. There is no obvious commitment of different cells to different enzymes and it is assumed that all the acinar cells synthesize all the enzymes characteristic of the exocrine pancreas, i.e. amylase, lipase, ribonuclease, trypsinogen, chymotrypsinogen, procarboxypeptidase A and B, trypsin inhibitor and proelastase (Rutter *et al.*, 1968a). Within any one acinar cell the different enzymes can be found to be concentrated in certain areas, ribonuclease with the golgi apparatus, the trypsinogens with rough endoplasmic reticulum and most of the others with the zymogen granules.

There are two main observations or deductions emerging from the work on exocrine pancreas which are of particular relevance to our topic of controlled gene expression. The first concerns the possible synthesis of these enzymes in most or all tissues of the mammalian body. In other words, are the genes, coding for these enzymes, completely shut down in non-pancreatic tissue, or is it merely a matter of quantitative control, so that they are synthesized in particular abundance in the exocrine pancreas? There are clearly great and sometimes insurmountable difficulties attendant on finding satisfactory experimental answers to such a question. The lower the concentration of a protein, the greater is the likelihood that its presence is the result of transport from elsewhere, or of lack of specificity in the assay procedure. Moreover, low concentrations do not lend themselves to ready characterization to allow comparison with the protein synthesized by other organs. However, although some of the products of these acinar cells are certainly not specific to pancreas, e.g. ribonuclease, others such as lipase and perhaps amylase are, in their pancreatic form, probably uniquely produced in these pancreatic cells. Rutter *et al.* (1968) detected two different lipase enzymes in the developing rat pancreas, styled lipases A and B, which were not detected in tissues other than in pancreatic tissues. Extremely low levels of these enzymes were detectable in the proto-differentiated exocrine pancreas and the assay system employed would indicate that the concentration regarded as absence would give less than 10 molecules/cell for non pancreatic tissue. The extremely sensitive assay for insulin permits the absence level of non pancreatic tissue to be placed at less than one molecule/cell. A clearer answer becomes apparent to this question of tissue specificity for enzyme synthesis in the discussion on insulin synthesis by the endocrine pancreas in the next section.

The other conclusion which can be reached from studies on the exocrine pancreas relates to the biphasic pattern of enzyme synthesis during pancreatic development. If assays for these enzymes are carried out throughout the differentiation of the pancreas, either *in vivo* or *in vitro*, an interesting pattern emerges in that rather low concentrations are found to be present in the secretory cells for some days, followed by a greatly increased synthesis during the most dramatic phase of cellular differentiation. This very high rate of synthesis during differentiation may be actually slightly reduced when the steady state stage of the fully differentiated organ is achieved. For example chymotrypsin, estimated as the number of molecules/acinar cell, gives values of 8×10^4 molecules in the early stages of differentiation, 3×10^9 molecules during the overt differentiation and 4×10^8 molecules in the steady state of the mature organ (Rutter *et al.*, 1968a). Rutter has assigned the term protodifferentiated to the first stage, differentiated to the second stage and modulated to the final stage when, of course, the mature organ does modulate its secretory activity in response to many internal and external factors. If the patterns of increasing enzyme synthesis during pancreas differentiation are compared, the curves are found to be slightly out of phase with one another. This may be due to actual uncoordinated synthesis of the various enzymes within the cells, but it is not possible to completely exclude other less interesting explanations such as differential breakdown or leakage from the tissues.

This pattern of protodifferentiated low-level synthesis, followed by a geared up production during differentiation proper, is interesting and important. It implies that the responsible genes begin transcription during the first stage but that their fully productive potential is not realized until later. Obviously, various possible post transcriptional steps, involved in the accumulation of the necessary messenger RNA and ribosomes before full scale production, can be visualized. This question has been partly explored by Wessels and Wilt (1965) by using 10^{-8} M Actinomycin D to prevent RNA synthesis. These authors found that if the drug was applied after the cessation of cell division in the differentiation of the pancreas, then, despite a 70% inhibition in RNA synthesis, there was no effect on zymogen granule formation or amylase production. If, however, the drug was applied during pancreas differentiation, then amylase synthesis and zymogen granule formation were almost completely inhibited. Because of the particular sensitivity of ribosomal RNA synthesis to the drug and the other side effects sometimes associated with exposure of cells to actinomycin D, it is perhaps desirable that these experiments should now be repeated and assays of other pancreatic enzymes included.

Rather similar experiments with actinomycin D and *in vitro* cultured rat pancreas rudiments have been published by Rutter *et al.* (1968b). Concentrations of 0·01 μg/ml (10^{-8} M) were applied at different periods of time during the differentiation of the pancreas rudiment and the effect on the synthetic rates of the different proteins analysed by H^3 leucine incorporation. The chief finding was of differential effects on different proteins, interpreted as indicative of sequential synthesis of relatively stable messenger RNA molecules.

2. Endocrine pancreas

This portion of the pancreas, as we have already mentioned, contains two populations of cells devoted to the production of two particular proteins, glucagon and insulin. These two distinct population of cells not only synthesize different endocrine hormones and are of distinct intracellular morphology but their embryonic development is quite distinct. The A cells are those which synthesize glucagon, a small protein of only twenty nine amino acid residues and, in the adult mammal, these cells may occur in the gut lining as well as in the pancreas (Forssman *et al.*, 1969). Moreover, even at the first appearance of the pancreatic bud in the embryo, fully differentiated A type cells are present and functional in glucagon synthesis. This clearly suggests a role for glucagon in early embryonic life and perhaps even in pancreas formation (Picket *et al.*, 1972). Due to the early differentiation of the A cells and the relative insensitivity of the assay for glucagon, it is not possible to say whether glucagon synthesis is, like that of the exocrine products, biphasic during development.

The B cells of the pancreatic islets are known to be the sites of synthesis of the important hormone insulin. This is a protein of molecular weight 5800, comprising two polypeptide chains A and B held together by disulphide bonds. Actually, the initial structure, proinsulin, is slightly reduced in size by the action of trypsin, to give the final active form of the hormone insulin. Perhaps the greatest attraction of insulin synthesis as a study in genetic control mechanisms is the great sensitivity of the insulin assay, as developed by Rutter *et al.* (1968a). The assay involves two immune reactions carried out in series and is a modification of the method of Morgan and Lazarou (1963). In this method, two immune reactions are carried out in series. In the first, insulin is bound to guinea pig anti-insulin antibodies, the conjugate forming a soluble complex. In the second, this insulin-anti-insulin complex is bound to rabbit antibody which has been raised as anti-guinea-pig serum antibody in the rabbit. This final complex is insoluble and comes down as a precipitate. By including radioiodine labelled insulin in the samples during complex formation, the extracted insulin being assayed will compete with the radioactive insulin for the antibody binding sites. Thus the radioactivity of the supernatant will give a measure of the unbound radioinsulin and therefore of the non radioactive insulin bound. A convention is adopted by which it is assumed that displacement of 50% of the radioactive insulin is effected by an insulin level equivalent to that of the radioactively labelled insulin. In fact this is not always the case, but test dilutions tend to yield a curve which will run through the point equivalent to a 50% displacement.

These authors have also ensured that high concentrations of non-insulin protein do not interfere with the assay, since, particularly in the early embryonic pancreas, the insulin concentration is very low as compared to that of other cellular protein. This modification of the original Morgan and Lazarow immunoassay for insulin is extremely sensitive, and its main limitation is simply that imposed by the maximum radio-iodination of the insulin. Approximately 10^6 molecules of insulin can be detected and so, by including one million or more cells in the assay, levels of about one molecule/cell may be theoretically detected. Some questions remain to be answered, unequivocally, about the assay at such dilutions, such as the interference by non-insulin molecules and the effectiveness of the assay for different insulins (the assay seems to detect proinsulin and any intermediates, as well as insulin, and is probably also fairly reliable for the different types of insulin molecules reported to occur in some species).

In any event, we have here what is certainly the most sensitive assay yet devised for any one protein synthesized by and present within cells. We can now proceed to ask what answers this highly sensitive test provides to the pertinent question of gene expression and its control. First, the insulin levels in non-pancreatic tissues appear to be almost zero; less than one millionth of the concentration in the pancreatic cells. Since insulin is conducted round the body as a hormone, these levels are probably correctly ascribed to diffused or transported insulin. This leaves the conclusion that controlled gene expression for this protein may be so stringent that absolutely no proteins are translated for the insulin genes in non pancreatic tissues. Moreover, in the differentiation of the pancreas itself, no insulin could be detected in the embryonic rat pancreas prior to day 10 of gestation. Following day 10 of gestation and up to about day 12 there is a rise in insulin concentration, then a plateau for about two days, followed by a dramatic rise in insulin accumulation up to about day twenty. This then is the characteristic biphasic pattern of synthesis already noted for exocrine pancreas differentiation. If the developing organ is carefully examined microscopically (Picket *et al.*, 1972), no sign of B cells can be found until about day 15 or 16, from which time substantial numbers can be recognized by the characteristic morphology of their secretory granules.

The picture of B cell differentiation which emerges is, therefore, that frequently stressed by Rutter. At first there is a pre-differentiation phase, when no B cells can be found and no insulin synthesis detected. Then a protodifferentiated phase ensues, when, despite the continuing absence of recognizable B cells, a rapid but low level rise in insulin production occurs. Clearly, this synthesis is occurring in cells not yet morphologically distinguishable as B cells and still in the process of differentiating. This is now followed by the dramatic rise in insulin concentration. Such an increase could, of course, be due either to a rapidly increasing number of B cells all making insulin at about the same steady rate, or to a great increase in the rate of insulin synthesis by a more or less fixed number of truly differentiated cells. Clark and Rutter (1972) have good evidence that the second explanation is the correct one, first, because of the lack of recognizable B cells in the early days of insulin production and second, because there is no great increase in the number of recognizable B cells during the period of accelerated insulin production. A 200-fold increase in the insulin concentration is matched by only a two- or three-fold increase in overall cell number and no proportional increase in B cell numbers in the endocrine tissue. Some further investigation of this question is desirable and is no doubt at present under way. It seems especially necessary to eliminate other factors, such as altered degradation rate and the possible reduced reactivity of the assay with proinsulin. Expression of the data in terms of rates of insulin synthesis rather than levels of accumulated insulin would also seem very desirable. Nevertheless, better than any other system, insulin synthesis in the B cells of the endocrine pancreas provides a picture of the controlled expression of one or two genes during tissue differentiation. The picture which emerges involves (a) stringent control of gene expression to prevent any specific protein synthesis prior to proto-differentiation, (b) a turned-on gene expression early in differentiation but tuned to a relatively low level of production of the specific protein and (c) a great elevation in the rate of production and/or accumulation of the specific protein as the relevant cells become completely committed to the differential destiny. A final point to emerge is (d) the modulated plateau level for the fully differentiated gland which is perhaps best understood as a dynamic balance between specific synthesis, export and specific degradation.

3. Comment

Studies on pancreas differentiation have made two great contributions to our understanding of gene expression. First, with extremely sensitive assays for lipase and insulin, the experiments suggest that genes said to be unexpressed in certain tissues may, in at least some cases, be completely unexpressed and absolutely no proteins may be made from those particular templates. This points to the possibility of extremely tight transcriptional control mechanisms. Second, the pattern of synthesis of many different enzymes during pancreas differentiation gives a biphasic pattern of production suggestive of different cell states, the protodifferentiated and the fully differentiated states.

Apart from the obvious suggestion that assays for other proteins should be modelled on the lines of the insulin detection system, say for crystallins, melanin and myosin, it also seems possible that the great sensitivity of the insulin assay could be utilized in other ways. What about the exposure of other cells to preparations of pancreatic protein or RNA, followed by insulin assays in the exposed cells? The added protein and RNA would themselves require to be exposed to an insulin assay and a time course curve of possible insulin accumulation in the experimental cells constructed, in order to preclude the obvious possibility of the passive transfer of insulin with the protein or RNA fraction.

B. Enzyme synthesis during the yeast cell cycle

Yeast is an organism which, like both types of slime mould, is a eukaryote with many of the cultural advantages of the prokaryotes. Yeasts may be grown easily in very large quantities, thus permitting sensitive assays for enzymes and nucleic acids. Moreover, yeast cultures can be synchronized, permitting study of the pattern of synthesis during the cell cycle. Although the synchrony of cultured yeast cells is experimentally imposed or selected, and is, therefore, less complete than the natural synchrony of *Physarum*, nevertheless, it is a synchrony of separate cells as distinct from the syncytial synchrony of the latter organism. Yeast cells do differentiate in that they may enter a meiotic rather than a mitotic cycle, culminating in sporulation. A recent review on the differentiation aspects of yeast sporulation will be found in Tingle *et al.* (1973). As a model of differentiation, yeast sporulation is probably less ideal than the spore production of *Dictyostelium* or *Physarum* and its biochemical basis has been less thoroughly studied. We will, therefore, omit further discussion of that aspect of yeast biology and apply our attention instead to the mitotic cell cycle in vegetatively growing yeast.

The great attraction of synchronized yeast cultures, for our purposes, is that they represent a very large population of essentially identical cells, all in phase as they proceed through the cell cycle. Such a culture situation can be expected to shed light on an extremely important biological question, namely how is gene expression modulated during vegetative growth? Given that changes in gene expression occur as cells differentiate, it is also important to look at the finer aspects of gene control during the stages of one cell cycle, without the complication of differentiation. As a cell passes through the stages of G_1, S, G_2 and mitosis, there will clearly be alterations in demand for different gene products, not only those associated with DNA replication and mitosis, but others which may be open to some regulation of synthesis depending on the availability or otherwise of nutrients in the media. Yet another factor will complicate any picture of gene expression which emerges from such studies—the doubling of the gene dose during S. This might be expected to lead to a doubling in the rate of synthesis of uncontrolled proteins with, perhaps, no change in the rate of synthesis of tightly controlled proteins.

The method of synchronization of yeast cell cultures merits some attention in this chapter, since the interpretation of the results is very dependent on how complete and authentic the synchrony is. At one time yeast cells were synchronized by the administration of starvation shocks or by exposure to agents which would block mitosis. Such methods are far from ideal, chiefly because there is some reason to doubt the normality of the resulting population when free from the restriction imposed by the physiological shock or drug. Of course, an alternative approach is to study single cells as they pass through the cycle, but all the advantages of bulk cultures and enzyme assays are then lost. Probably the most reliable method of obtaining synchronous cultures of yeast cells is that of cell selection. Both budding and fission yeasts alter in size as they pass through the cycle, a most fortunate aspect of cell biology. This permits the concentration of cells from an experimentally growing culture and their subsequent centrifugation through a gradient of sucrose in a conventional or zonal rotor. Small cells, which are at the beginning of the cell cycle, move slowly through the gradient and may be separated off to act as the initiators of a synchronous culture. Such methods have been used by Mitchison (1973a) for the fission yeast *Schizosaccharomyces pombe* and by Halvorson *et al.* (1971) in the budding yeast *Saccharomyces cerevisiae*. A different approach is to select cells from an exponential culture on the basis of size, using a zonal rotor, and to utilize the different size classes obtained as populations of cells in different stages of the cycle, assaying the cultures without further growth. Such a method avoids the recovery of cells from the insult of the cell selection procedure but, as with gradient selection, it fails to distinguish cells which are of similar sizes but rather different stages of the cell cycle.

Another reason why the method of synchrony is so important to the interpretation of the experiments is that incomplete synchrony will greatly blur the pattern of the synthesis curves. If, in a perfectly synchronized population, two quite separate peaks of synthesis occur, with a marked trough between, a poorly synchronized culture might blur out the trough to show a rather steady state of enzyme production.

It is also crucial to understand what is being measured in such experiments. It is the amount of availably active enzyme within the cell population at the stated time. The results are almost always obtained by devising an assay which detects the amount of enzyme on the basis of its activity. It follows that certain assumptions are inherent in deducing information about synthesis, especially about mRNA synthesis, from such experiments. Almost invariably, workers in this field have assumed a steady rate of enzyme degradation, or, on the other hand, no degradation at all. Now this is not always a satisfactory assumption. Assuming that, in one set of four measurements at different times in the cycle, a particular enzyme gives activity values of 2, 4, 6 and 8, with no degradation, this would mean a steady production of 2 units of enzyme/timed interval throughout the cycle. On the other hand, it could mean that rapid degradation occurred in the third interval but synthesis was raised to allow for the increased degradation, so giving an apparently steady rate. To take another hypothetical set of results, figures of 2, 4, 2 and 4 could be assumed for the enzyme activity at similar intervals. Here there has clearly been degradation (or sequestration resulting in inactivity) between the third and fourth interval. What we do not know is what the rate of synthesis was. Did it change, or did it remain constant?

There are a number of ways of reducing the seriousness of the above objections: First, by taking very frequent measurements and so reducing the length of the intervals and second, enzyme activity may be followed after the addition of an inhibitor of protein synthesis, such as cycloheximide or puromycin (Mitchison and Creanor, 1969). Until such times as more is known about the specific breakdown of enzymes and their effective neutralization (see discussion in Tomkins *et al.* (1969) and Rutter *et al.*, discussed in this book in Section 4A), it is probably justifiable to assume that synthesis is more tightly controlled than degradation and that peaks of enzyme activity truly reflect peaks of enzyme synthesis.

A related problem with the experimental procedure is that enzyme activity and not total enzyme protein is measured. Clearly, changes in activity could reflect changes in the levels of associated activators or inhibitors. Our present knowledge of the widespread occurrence of zymogen forms of enzymes would certainly encourage caution in this area. This question has been discussed by Mitchison (1973a) who points out that the use of an immunological detection system would obviate this objection. Such a technique does, however, raise other and perhaps more serious problems of cross reaction. As with the difficulty of specific degradation, it seems unlikely that specific enzyme activation or inhibition does, of itself, account for the shape of the activity curves obtained. It is, however, a biochemical phenomenon which should not be overlooked in this context.

Having stressed the advantages and weaknesses of the methods, what are the results of the observations? A most comprehensive tabulated account of such experiments, not only in yeasts, but in other eukaryotic and in prokaryotic cells, is given by Mitchison (1971). He distinguishes three patterns of enzyme synthesis, step enzymes, peak enzymes and continuous linear enzymes. Step enzymes are those which display a distinct doubling during one stage of the cycle, as does, for example, the DNA. Peak enzymes are those which display one or more peaks of synthesis during the cell cycle, followed by rapid enzymic inactivation or degradation. Continuous enzymes show a variety of patterns which are basically either exponential increase curves or of a linear rate of synthesis with an angle in the line where the rate of synthesis doubles. It is very important to stress again that what is being measured in these various patterns of enzyme synthesis is rise and fall of enzyme activity, not rate of synthesis. To that extent, a linear curve may represent a constant balance between synthesis and degradation while a peak very probably represents synthesis followed by degradation. This point deserves to be recognized more often in discussions of these phenomena. In general, however, it is likely that the linear enzyme curves are the result of synthesis, being closely related to the replication of the genome, with little or no modulation at a transcriptional level necessary. On the other hand, step and peak enzymes are likely to involve some control of synthesis, degradation or both. Even step enzymes, which might be thought to be rather simply related to gene dose and DNA doubling, often continue to show a step pattern when DNA synthesis is blocked (see discussion in Mitchison, 1971). Of 22 enzymes in *S. cerevisiae*, one in *S. dobzhanskii*, three in *S. dobzhanskii* × *fragilis* and nine in *S. pombe*, all are step or peak enzymes with the exception of four. A majority of enzymes in other synchronized eukaryotic cells are also seen to be step or peak enzymes in Mitchison's table. Mammalian cells tend to show more continuous linear enzymes than do the lower eukaryotes or prokaryotes.

Of course, such curves of enzyme levels in the cell involve another complicating variable, that of enzyme induction. The curves discussed above are all constructed from cell cultures maintained in a relatively steady state of nutrient availability. But it is also possible to remove cells from such cultures and test the response in levels of particular enzymes, following their specific induction or derepression. Such a response might also be expected to vary at different periods of the cell cycle. The rate at which enzyme activity rises in the cell population is termed the potential of that enzyme at that stage of the cycle. Experiments by Mitchison and Creanor (1971) on sucrase and maltase induction in *S. pombe* clearly demonstrate that potential increases are stepped.

The step occurs shortly after the S period, however, and is perhaps a gene dosage effect. This contrasts with the curves for truly stepped enzymes, where the step is not co-incident with the S period and where the stepped pattern will present itself even when DNA synthesis is blocked (Mitchison, 1973b).

The key question in gene expression for which yeast enzyme synthesis provides some clues is how are the levels of peak and step enzymes regulated during the cell cycle? This is probably a part of the more general question of how gene switching is accomplished in eukaryotes. Two main theories have been presented, which can be examined in the light of the yeast evidence. The first is that of oscillatory repression, originally suggested as a mechanism regulating prokaryote enzyme synthesis by Donachie *et al.* (see Donachie and Masters, 1969). This theory assumes that enzymes are subject to repression by their end products as a negative feed back mechanism. Such feed back will, in most conditions, lead to oscillation of synthesis and repression and it is assumed that these come to be locked-in with steps in the cell cycle of the organism. Of course, in conditions of complete repression, no synthesis might occur at all and in conditions of complete induction or derepression, the enzyme synthesis curve would be a continuous linear one.

The second and alternative theory has been promoted by Halvorson *et al.* (1971) and has come to be termed linear reading. In brief, this theory proposes that genes are transcribed in the same linear sequence as their order on the chromosome. This theory would assume that a very limited number, perhaps only one, of RNA polymerase molecules would traverse the genome during any one cell cycle and that a gene would only be transcribed for a brief part of the entire cycle. The theory draws support in prokaryotes from work on *Bacillus subtilis* (Masters and Pardee, 1965) and in eukaryotes from work on budding yeast, *S. cerevisiae* (Halvorson *et al.*, 1971). In a study of four different enzymes in the budding yeast, the timing of the steps was consistent with a linear reading from one end of the chromosome to the other. These four enzyme genes are on the fifth chromosome and are termed ur_3, th_3, hi_1 and is_1; all peak in the order in which they are known to occur on the chromosome. In an actual measurement of the timing correlation, Cox and Gilbert (1970) made the interesting observation that two *S. cerevisiae* enzymes, galactokinase and α amino-adipic reductase, which have peak expressions 0·15 of the cell cycle apart, are 50 centimorgans apart on chromosome 2. Moreover, in another strain of this yeast the two enzyme genes are much further apart on the chromosome and so too are the two steps in the cell cycle.

These data do not amount to absolutely convincing evidence for linear reading, but they are certainly persuasive. As concluded by Mitchison (1971) it is quite probable that both mechanisms operate in eukaryotic cells. Yet other mechanisms may be additional to these.

Whatever mechanism or mechanisms operate to control gene expression in eukaryotes, one of the really interesting and curious aspects of the present data on enzyme synthesis during the cell cycle is the large number of enzymes which are synthesized periodically as step or peak proteins, rather than continuously. It is hard to imagine that, from the point of view of the cells' metabolic machinery, it is an ideal system. There is some indication from the data (Mitchison, 1971) that continuous linear enzyme synthesis is more frequently found amongst higher eukaryotic cells rather than in yeasts and algae. Could this be an evolutionary development? It is also hard to see how enzymes, operating under oscillatory repression, could have their genes interspersed with those of other proteins, controlled by linear reading. It will be interesting to find whether, as more information becomes available, genes controlled by the two separate mechanisms turned out to be grouped separately in the genome.

1. Comment

It is unlikely that the widely spaced and definitive genetic events of major differentiation or development are at all analogous to the modulation of gene expression which occurs in the cell cycle.

But, equally, the day to day life of cells, differentiated or otherwise, does involve changes in gene expression which are possibly often regulated at a transcriptional level. Responses of mammalian cells to circulating hormones must certainly be such an example. Probably, no eukaryotic system has proved as useful in this regard as the yeast cell cycle which is yielding some hard data on which relevant theories can be based. It, therefore, seems all the more desirable that further evidence for linear reading mechanisms should be sought from synchronized yeast cultures, together with some attempt to map genes coding for apparently continuous linear enzymes. With oscillatory repression mechanisms, there is the interesting point of how they might come to be (as they are) geared in to the cell cycle itself. It seems, at least theoretically, likely that such a geared relationship could be broken without preventing the oscillations.

Finally, since so much of the evidence for oscillatory repression and linear reading, as eukaryotic genetic control mechanism, is drawn from work with yeasts, it seems highly desirable that more attention should be given to post-translational control mechanisms such as enzyme sequestration and degradation. The recent discovery of a good and selective inhibitor of yeast RNA synthesis, 8-Hydroxyquinoline, should prove very useful in this regard (Fraser and Creanor 1974).

C. Muscle

Muscle constitutes an important system for the study of gene expression, chiefly because a number of quite different proteins are specific to its cells. They are myosin, actin, tropomysin, troponin, three forms of actinin, the enzymes creatine kinase and muscle phosphorylase and the respiratory protein myoglobin (actually the extent to which these proteins are exclusively possessed by muscle cells is a matter to be discussed later). In addition to these proteins, lactate dehydrogenase enzymes have a characteristic isoenzyme distribution in muscle, but this is discussed further in Section 3B.

Since muscle is a rather complex tissue of wide distribution in the animal body, its biochemistry is also complex. Good references to the biochemistry of muscle structure and function are Needham (1971) and Lehninger (1970). It is customary to subdivide muscle tissue histologically into cardiac, smooth and striated muscle and the three tissues differ somewhat in structure and function. There is some evidence that the actin and myosin of cardiac muscle differ from that found in striated muscle, but the experimental evidence is not very extensive. Cardiac muscle tissue is made up of individual muscle cells, and the myofibrils within are enveloped by a poorly developed sarcoplasmic reticulum. Smooth muscle, like cardiac muscle, is cellular in structure and contains actin and myosin of the same form as that found in striated muscle, but in a much lower concentration (Needham, 1971). One protein has been stated as specific to smooth muscle, namely the form of myosin found in the tonoactomyosin, now often termed tonomyosin. It has been suggested that this form of myosin is similar to that of cardiac and striated muscle, but with an added third component (Hamoir, quoted in Needham, 1971), but artefacts following from extraction procedure may account for this. Cardiac and smooth muscle are obviously difficult tissues on which to carry out basic biochemical work and much remains to be done. These will, therefore, be left aside here and we will concentrate on the more complicated, but better understood, striated muscle. Striated muscle is often termed skeletal muscle since it is associated almost entirely with the skeletal structure, while smooth muscle is found lining the walls of gut, uterus, blood vessels and other non skeletal tissues and organs. Simple dissection will reveal that skeletal muscle often varies in appearance and may be broadly categorized as red or white muscle. Fish muscle, for example, is characteristically white, while the muscle of pigeon breast is noticeably deep crimson in colour. The actin and myosin molecules of the two tissues are probably identical but there is great variation in the tissue metabolism. White muscle has a high capacity for the use of glycogen and a low oxidative metabolism, while red muscle has a high oxidative potential. Differences in the roles of the two types of muscle are also demonstrated by their distribution and activities, white muscle being associated with rapid phasic contraction and red muscle with repetitive or prolonged contractions. For our purposes the most interesting difference between them lies in their myoglobin content, which is low in white muscle and high in red muscle, and largely accounts for the marked difference in colour.

1. Structure of skeletal muscle

Skeletal muscle is composed of numerous fibres, each fibre being a syncitial structure with, often, hundreds of nuclei. These structures are the result of the fusion, during development, of the single muscle cells, the myoblasts. Within the syncitial cell groups are the parallel contractile elements, the myofibrils (see Fig. 7), while the surface of the muscle syncitium is termed the

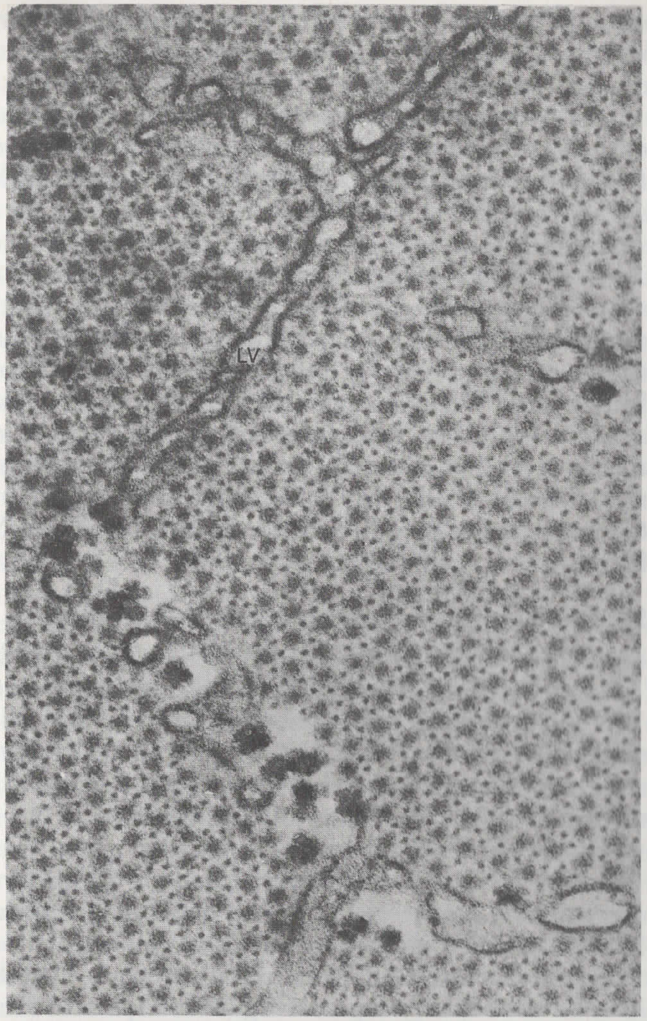


FIG. 7. Electron micrograph of part of a frog skeletal muscle cell showing many myofibrils in cross-section. Note the regular assay of six thin filaments about each thick one. LV marks sections of longitudinal vesicles running between the myofibrils. Each complete myofibril is about 1 μ in diameter (kindly provided by Dr. H. F. Huxley).

sarcolemma. Within the syncitial fibres is an intracellular fluid which bathes the myofibrils and this is termed the sarcoplasm. This is made clear when muscle proteins are extracted. When a water extract of skeletal muscle is made, the intracellular enzymes are removed, including the creative kinase, phosphorylase, and myoglobin already alluded to. Only extraction with 0·6M KCl will solubilize the contractile proteins of the myofibrils, containing the actomyosin complex and the other associated filamentous and cross-linking proteins. Muscle cells also contain two extremely elaborate membrane systems, referred to as the transverse tubular system and the sarcoplasmic reticulum. They consist of a sleeve-like system of vesicles enveloping the bundles of myofibrils and the electrical responses of the transverse tubular system are closely bound up with the translation of an electrical signal from a nerve cell into contraction of the myofibrils with the muscle syncitium. A thorough and scholarly account of the structure of the sarcoplasmic reticulum will be found in Porter (1961).

2. The development of skeletal muscle in the embryo

The myoblast cells which ultimately fuse to form the syncitial fibres of skeletal muscle are developed initially from embryonic mesoderm. Sequences in the development of myoblasts are discussed by Herrmann *et al.* (1970). In the chick limb bud, evidence for myosin synthesis has been obtained from stage 24 embryos. This is rather later than the appearance of chondroitin sulphate, suggesting that cartilage stabilization may precede that of muscle in these limbs. Overt myogenic development in the limb bud is initially paralleled by intense cell proliferation of the myogenic tissue, cell division reaching a peak on the fifth to seventh days of development, by which time some 70% of all the myogenic cells are rapidly replicating (Marchok and Herrmann, 1967). After the seventh day, cell division in the chick leg muscle rapidly declines (Herrmann *et al.*, 1970) so that by the 11th day of development only 25% of the myogenic cells are replicating. As we have commented earlier, the mature muscle fibre is a syncitium which derives from the fusion of many myoblasts. This fusion occurs soon after the decline in myogenic cell proliferation, but with a lag of only a few days. Most of the cell fusion is complete by the 18th day of chick development. Fusion of myoblasts has been followed *in vitro* (Yaffe, 1969) as well as *in vivo*.

A very neat experiment has been executed to demonstrate the fusion of separate myoblasts *in vivo* and also to establish that blocks of muscle tissue are derived from more than one basic developmental clone. The experiment involved forming chaemeric mice by blastomere fusion between two mouse strains with differing isocitric dehydrogenase enzymes. This enzyme is a polymer of two subunits, the subunits being expected to form a new class of hybrid enzyme molecules in the muscles of the chaemeric mice: Such a result was in fact obtained (Mintz and Baker, 1967).

Although most of the cells within the developing myogenic tissue fuse together to form fibres, some apparently identical cells continue to divide slowly throughout the life of the organism, acting as a supply source for new myoblasts. The steps of muscle differentiation can all be followed in tissue culture, although the timing of the steps is not identical to the *in vivo* situation. This distinction has been utilized and exploited in order to separate the phases of cell proliferation and cell fusion and synthesis of the characteristic muscle proteins can be assayed in the two phases of myogenic development (Konigsberg, 1963 and Coleman and Coleman, 1968). Of course smooth muscle cells do not undergo fusion and the programme of synthesis of muscle proteins is somewhat different to that found in skeletal muscle. Unfortunately, very little work has been done on the biochemistry of differentiation in smooth and cardiac muscle.

Since embryonic myoblasts, prior to fusion, will readily grow in culture, it has proved possible to study the stability of their determination by repeated subculture. Provided that the cells are subcultured before they grow to confluence—if they are permitted to become confluent the myoblasts may begin fusion—the same cell line may be maintained for many months. At any time during culture, a plate of cells may be permitted to grow on and the morphological and biochemical signs of terminal myogenesis scored. When this experiment was maintained over a two year period, all cells continued to express only a myogenic cell type of determination and would only develop into muscle cells (Yaffe, 1969). As with the *Drosophila* imaginal disc cell (see Section 5F), the embryonic myoblast maintains its determined state over many generations.

The biochemical parameters of muscle differentiation will be discussed under the headings of the different molecules involved, but one point which emerges may be picked up here. It is that, although some indications of the synthesis of muscle specific proteins may be detected in pre-fusion myoblasts, the large scale production of muscle protein does not occur until cell proliferation of the myogenic cells has largely run down just prior to fusion. This has led those working on muscle differentiation to suggest a conflict between cell division and terminal differentiation. Although the thesis is, in broad terms, probably correct, it is certainly untrue in its extreme form, as will be discussed elsewhere. Differentiated blood cells (Thomas and Maclean, 1974, 1975) rich in haemoglobin, are capable of division. So, too, are well differentiated heart muscle cells (Goldstein *et al.*, 1974).

Another aspect of the embryology of muscle which must not be overlooked is that embryonic muscle tissue consists of at least two cell types, myoblasts and fibroblasts. It is, therefore, imperative to consider the influence of the fibroblasts on the myoblasts and on myogenesis. Hauschka (1968) has attempted to explore this relationship and concludes that the synthesis of collagen by the fibroblasts is a frequent, if not an invariable, prerequisite for myoblast development and fusion. Since collagen is exported by fibroblasts, it seemed reasonable to investigate the influence of collagen on myoblast differentiation and Hauschka has found that, although myoblasts may be determined prior to exposure to collagen, their overt differentiation depends on contact with this collagen. Clearly, a system which makes the release of one genetic programme dependent on the product of another different programme must be a frequent device in the organization of morphogenesis.

3. Muscle cell proteins

(a) *Myosin*

The water insoluble filamentous elements of skeletal muscle are made up to two main proteins, myosin and actin. Myosin and actin were originally extracted together as a complex called actomyosin, but this material is now recognized to be an aggregate of actin and myosin. The complex is also sometimes referred to, somewhat confusingly, as myosin B. Together these two proteins make up about 80% of the contractile protein of the muscle elements and individually myosin accounts for 54%, actin for 25%. The remaining 20% of the contractile apparatus consists of the minor components tropomyosin, troponin and actinin.

Myosin is a protein of about 470 000 molecular weight and actually consists of two identical polypeptide chains which are wound together for most of their length. Each chain is made up of about 1800 amino acids. Although the greater part of the myosin molecule is, therefore, a double helix of the two polypeptide chains, at one end the two chains are free, each end being folded into a separate globular structure. These globular ends also contain another two or three extra small polypeptide chains in their structure (see discussion in Lehninger, 1970). These globular complexes at the head of the myosin molecule are of great importance in its activity since they seem to be the sites not only of the ATPase activity of the molecule, but also of its binding with the other major muscle protein, actin.

For many years, myosin (or actomyosin) was looked on as a protein unique to muscle tissue. Today, the picture is much less simple. Not only can myosin-like proteins be extracted from the myxomycete *Physarum* and the isolated cilia of the ciliate *Tetrahymena pyriformis*, but such proteins can also be extracted from sperm tails and even from mitochondria. The evidence is often contradictory (see discussion in Needham, 1971). The most satisfactory attempts at testing the identity of such molecules have so far come from immunological assays. We have already noted that the myosins of smooth and fast skeletal muscles are immunologically distinct (Finck, 1965). Moreover, antisera made against chicken skeletal muscle myosin gave negative results with extracts of chicken ciliated epithelium and sperm. Perhaps of passing interest is the observation that antisera raised against human smooth muscle myosin gave a positive reaction against thrombothenin, a protein extracted from aggregates of blood platelets (Groschel-Stewart, 1969). Whether this means that smooth muscle cells or molecules have become incorporated into the megakaryocytes during their development (platelets are non-nucleated cytoplasmic fragments of megakaryocytes) or that these cells normally synthesize an identical myosin, it is impossible, for the present, to judge.

(b) *Actin*

This protein is much smaller than myosin, having a molecular weight of about 46 000. It may occur in two forms, one being a polymer of the other. G-actin, or globular actin, may exist as a monomeric globular protein, but under certain conditions it polymerizes to form a double helical arrangement termed

F-actin. Many G-actin molecules are involved in the formation of the long double helix of F-actin, which makes up each thin filament. G-actin and myosin are conjugated in a ratio of 1 : 1 in the composition of actomyosin and, since the actin is normally in the F-form in the complex, actomyosin consists of a long rope of actin to which many myosin molecules are attached, one myosin head to one actin monomer in the rope.

As with myosin, the specificity of actin to muscle is now less certain. Present evidence, though not absolutely unequivocal, suggests that the actins of smooth and skeletal muscle are the same protein (Carsten, 1965). But actin has, like myosin, been extracted from *Physarum* and *Tetrahymena* cilia. Actin-like protein has also been obtained from preparations of flagellae from sea-urchin sperm.

There are now numerous reports of the isolation of actin-like molecules from dividing cells, nerve cells and eggs. It seems likely that the mitotic spindle of dividing cells has contractile properties and that these may be explained by the finding of this contractile protein. Miki-Noumura and Oosawa (1969) have isolated actin from sea urchin eggs which reacts with mammalian skeletal muscle myosin in an identical way to native muscle actin.

Clearly, the simple picture of actin and myosin being exclusively muscle tissue proteins must be abandoned. Since many cells have contractile elements within them as a normal part of their morphology—cilia, centromeres and perhaps microtubules—and during at least part of their cell cycle synthesize contractile protein for the mitotic spindle, the widespread, if not universal, occurrence of actin and myosin must be expected. Whether all these actins and myosins within any one species are products of the same genes is not clear, with most evidence pointing to a contrary conclusion. Nevertheless, the genes for actin and myosin may well be examples of genetic units which function extravagantly in one tissue muscle, and to a much more limited extent in most other cells for special parts of the cell cycle.

(c) *Tropomyosin*

This is another fairly major protein of muscle tissue, making up 11% of the contractile proteins. It has a molecular weight of about 130 000. Two different types of tropomyosin are known, of which the commonest, tropomyosin B, occurs in all muscles, while tropomyosin A occurs only in certain specialized muscles of molluscs. Tropomyosin B is a double helix of two identical subunits and so the complex resembles the tail portion of the myosin molecule. When mixed with F actin, tropomyosin B seems to occupy one groove of the F-actin double helix.

(d) *Troponins*

This is a protein of about 86 000 molecular weight which, together with tropomyosin B, forms a relaxing protein, which is also bound to the actin filament. Troponin binds calcium ions very tightly and seems to confer the calcium sensitivity to the muscle fibre complex.

(e) *Actinin*

Two proteins, α actinin and β actinin, are further minor components of the actin filament. There is now good evidence that α actinin is a conjugate of two dissimilar polypeptide chains.

(f) *Creatine kinase*

Energy for muscular contraction comes from the hydrolysis of adenosine triphosphate (ATP) and a particular compound, creatine phosphate, is stored in muscle to permit the regeneration of ADP to ATP. This reaction is catalysed by a special enzyme, creatine phosphokinase. This is a protein with a molecular weight of 80 000 and there is now evidence that there are different isoenzymes of this protein (Dawson *et al.*, 1968). Indeed, these authors have shown that there is an interesting succession of the form of the isoenzymes present in the developing muscle of foetal rat and chick. The three forms are styled 1, 11 and 111, the adult muscle possessing only the third form. Brains of adult animals were found to possess form 1 of this enzyme, indicating that it is not absolutely specific to muscle.

(g) *Phosphorylase*

Phosphorylase enzymes are involved in the conversion of glycogen to available energy yielding molecules. The enzymes are found both in liver and in skeletal muscle, but muscle and liver phosphorylases are believed to be genetically distinct. They can be distinguished immunochemically. The liver enzyme is stimulated by glucagon, the polypeptide hormone secreted by the α cells of the pancreas, but the muscle enzyme is refractory to the activity of the hormone both *in vivo* and *in vitro*. Liver phosphorylase is probably a dimer, while the muscle protein is, in its active form, a tetramer. Both the liver and muscle form of phosphorylase have been studied by Krebs and Fischer (1962). Although the evidence is far from watertight, it is likely that muscle phosphorylase is largely or entirely specific to that tissue.

The phosphorylase of skeletal muscle exists in two forms, an active form, phosphorylase a, and a much less active form, phosphorylase b. Phosphorylase a is known to have a molecular weight of 380 000 and to be a tetramer of four identical subunits. It has also been crystallized in pure form. There is a constant change in the muscle cell from one form to the other, mediated by two other enzymes, phosphorylase phosphatase and phosphorylase kinase. The shift in concentration of the dimer and tetramer varies the rate of conversion of the glycogen to glucose-1-phosphate. Actually, there is now evidence which points towards muscle phosphorylase being an allosteric protein, the combination of AMP with phosphorylase enzyme endowing the enzyme with an increased affinity for its two substrates, glycogen and inorganic phosphate.

(b) *Myoglobin*

This protein has become famous because of its use in basic studies on protein structure by Kendrew *et al.* (1960). It consists of a haem group in combination with a single globulin polypeptide chain. The latter consists of 153 amino acid residues with a total molecular weight of 16 700. The globin of myoglobin is distinct from any of those found in animal haemoglobins, although its similarity to the α globin chain of haemoglobin has invited the suggestion that they are the products of genes which may have evolved from a common original gene (Ingram, 1963).

Myoglobin is used to store oxygen in muscle and may exist as deoxymyoglobin, when the haem group is not occupied in the sixth position, or oxymyoglobin when this group is occupied by an atom of oxygen. Different muscles vary greatly in their content of myoglobin and this can be ascertained at a glance by the colour of the muscle. So white muscle has a very low myoglobin content, red muscle a high myoglobin content and the dark red or brown muscles of diving mammals—whales and seals—a very high myoglobin content. It should be noted, however, that individual muscle fibres within one muscle often vary in their colouration and that red muscle fibres are often located deeply within a mass of otherwise white muscle. A number of workers have sought for evidence of a foetal myoglobin distinct from the adult form, but the search has so far drawn a blank (Wolfson *et al.*, 1967). As the evidence stands at present, the globin of myoglobin is a gene product which is expressed only in muscle tissue and, within that tissue, its concentration is highly variable. It follows that the activity of this gene should prove to be of great interest in developing muscle, especially *in vitro*, and in situations in which muscle cells in culture are stimulated to grow in altered oxygen tension. In fact, myoglobin is a somewhat neglected molecule, aside from its intensive use for structural studies of protein architecture. A recent review of the molecule will be found in Kagen (1973).

4. Control of the levels of muscle proteins

Since muscle possesses many proteins, contractile, enzymic and respiratory, some at least of which are more or less tissue specific, it follows that muscle development and growth are interesting as models of transcriptional programming. It has already been emphasized in this chapter that perhaps no single protein has been really convincingly shown to be unique to muscle tissue, although some may be. The proteins associated with the contractile mechanism, although the ones most readily associated with muscle, may well be the poorest candidates for this role.

A detailed review of molecular synthesis during muscle development has been compiled by Herrmann *et al.* (1970). The relationship between the termination of myoblast proliferation, cell fusion and the synthesis of muscle proteins has already been alluded to. In the case of myosin, it has been studied by Holtzer and his associates (Okazaki and Holtzer, 1966). Myosin can be detected at 50 h development in brachial myotomes and four days in the limb buds of chick embryos, using a fluorescent anti-myosin antibody technique. However, this technique is estimated to be insensitive below a concentration of 1×10^5 myosin molecules/myoblast, and Medoff (unpublished, quoted in Herrmann *et al.*, 1970) argues that concentrations below this level are present for some time in the determined but still proliferating myogenic phase. Indeed, the four day levels in the chick limb bud are present in a stage of continued myogenic proliferation. A different picture emerges from the work of Paterson and Strokman (1972). It is that, prior to cell fusion in myogenic differentiation, no myosin whatever is synthesized. These authors were able to block cell fusion in embryonic explants with aminoethyl ether tetraacetic acid (EGTA). In explants so treated, no evidence of myosin synthesis could be found. A very low level of myosin was detected as being present and was attributed to very early fusion of some myoblasts, prior to the application of EGTA. If cell fusion was permitted to proceed in these cultures, a lag period of 8–10 h intervened between the burst of cell fusion and the burst of myosin synthesis. Paterson and Stokman (1972), therefore, believe that myosin synthesis does not begin until after cell fusion, i.e. that the developmental programme is tightly ordered and, in myogenic differentiation, fusion precedes myosin synthesis. Actually, a great deal rests on how the small amounts of myosin present, prior to fusion, are explained. It seems at least possible that in muscle, as in, say, pancreas, some low level synthesis of specific proteins occurs prior to the escalation following the onset of differentiation proper.

The synthesis of actin, although discussed less widely than myosin synthesis in developing systems appears to follow a similar course as does tropomyosin synthesis when assayed (Herrmann *et al.*, 1970).

Myoglobin synthesis has been followed during chick myogenesis by Kagen and Linder (1970). These authors prepared a radioactively tagged anti-myoglobin serum and exploited it for the detection of myoglobin in chick embryos. They were able to detect myoglobin in their earliest cultures—one-day-old embryos—and the synthesis of this protein rose sharply (12-fold) between the second and fourth day of development. Myoglobin synthesis varies greatly in different muscle types in the chick. Detectable first in the head and mid body regions, its synthesis in the skeletal muscle progresses slowly and continues well into adult life. In the cardiac muscle, however, although not detectable until the second day, its synthetic rate rises very rapidly and on day 2 of development the heart accounts for 25% of myoglobin synthesis. Kagen and Linder (1970) have compared myoglobin content with total soluble protein, finding that at one day old the myoglobin constitutes 7–8% of the total soluble protein of the embryo, but that by day 7 it accounts for only 1%. These figures do not so much represent a real fall in the rate of myoglobin synthesis, only a comparative fall. That is that the rate of synthesis of other soluble proteins outstrips that of myoglobin soon after day 1.

The appearance of other muscle proteins during development has been followed by a few workers. Yaffe (1969) noted that creatine kinase activity was slightly detectable prior to myoblast fusion but increased rapidly after fusion to a plateau on the 10th day of embryonic development. Phosphorylase synthesis and accumulation has been studied by Love *et al.* (1969), the main findings being that its synthesis lags behind that of myosin and that, at a time when myosin synthesis is slowing, that is during or

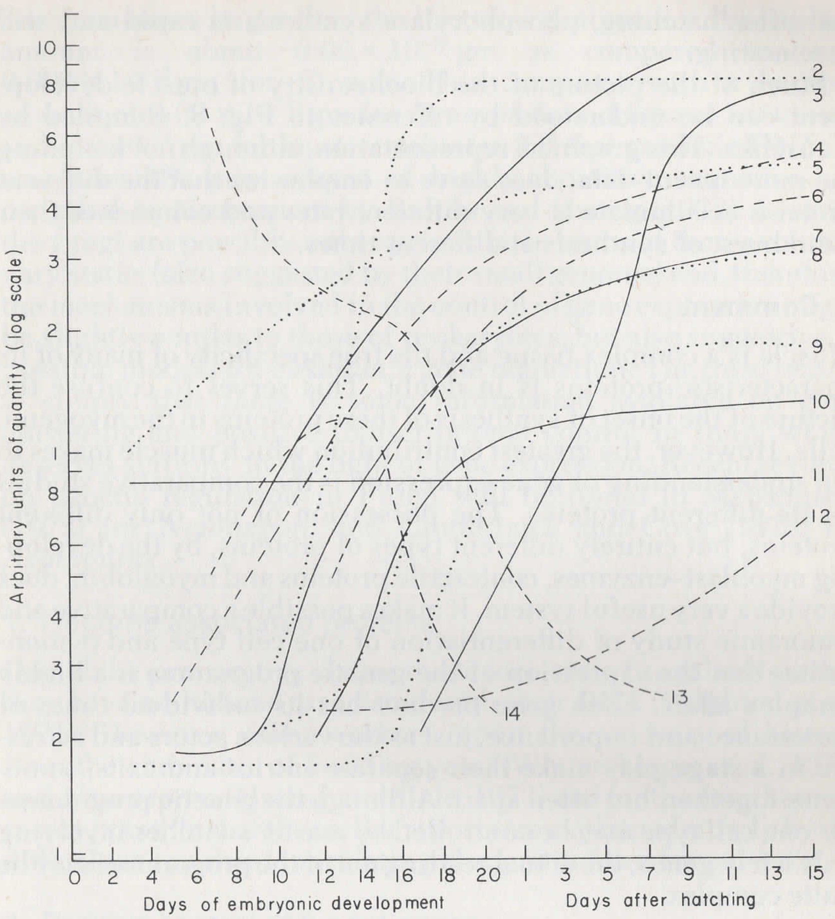


FIG. 8. A compilation of measurements showing the rates of change in levels of various biochemical and cellular parameters during the development of chick skeletal muscle. 1. myosin; 2. collagen; 3. phosphorylase; 4. creatine kinase; 5. aldolase; 6. phosphoglycerokinase; 7. glycogen; 8. actomyosin; 9. glutamate-oxalacetate transaminase; 10. adenylpyrophosphatase; 11. isocitrate dehydrogenase, hexokinase and phosphofructokinase; 12. alkaline phosphatase; 13. percent nuclei synthesizing DNA; 14. acetylcholinesterase (from Hauschka, 1968).

just after hatching, phosphorylase synthesis is rapid and still accelerating.

Much of the picture of the biochemistry of muscle development can be understood by reference to Fig. 8, compiled by Hauschka. This graphical representation, although not including the more recent data, does serve to emphasize that the different proteins accumulate at very different rates and commence their main burst of synthesis at different times.

5. Comment

Muscle is a complex tissue and the true specificity of many of its characteristic proteins is in doubt. This serves to confuse the picture of the onset of synthesis of these proteins in the myogenic cells. However, the greatest contribution which muscle makes to our understanding of gene expression is the comparative studies on its different proteins. The possession of not only different proteins, but entirely different types of proteins, by the developing myoblast–enzymes, contractile proteins and myoglobin, does provide a very useful system. It makes possible a comparative and panoramic study of differentiation of one cell type and demonstrates that the expression of the genetic programme is a highly complex affair. Each gene product has its individual times of appearance and importance, just as the various actors and actresses in a stage play make their separate entries and exits, sometimes together, but often apart. Although the genetic programme for one cell type may be controlled by master switches involving only a few genes, the actual reading out of the programme may be quite complex.

D. *Aspergillus* and *Neurospora*

This section will look at the results of experiments on fungal cells which yield information about the arrangement of genes on the genome and the mechanisms of gene activation and repression. We will not, therefore, attempt to review the very large volume of work on the general and biochemical genetics of these and other fungi. Fungal cells are of course eukaryotic, possessing a nuclear membrane system, and indeed the membrane persists during mitosis in this group. Fungal cells are of particular interest to us in terms of gene expression for a number of reasons. First, the size of their genome is much less than that of most eukaryotes, being some ten times larger than the bacterial genome, i.e. the haploid amount is about $0{\cdot}02 \times 10^{-12}$ gm as compared to, say, $0{\cdot}002 \times 10^{-12}$ gm for *E. coli.* This compares with a haploid amount of $0{\cdot}09 \times 10^{-12}$ gm for *Drosophila* and figures between 1 and 3×10^{-12} gm for most vertebrates. Of that genomic DNA, as much as 20% may consist of highly repetitive sequences, as recorded in *Neurospora* by Brooks and Huang (1972). Second, the fungi are possibly rather primitive in terms of their evolutionary status (also suggested by their small genome) and, therefore, the mechanisms involved in the control of gene expression might be slightly similar to those of prokaryotes, but also suggestive of possible models for eukaryotic gene regulation. The third reason is simply that there is some information available on gene clustering and positive transcriptional control in fungi which deserves scrutiny in the light of gene expression. Recent reviews on genetic regulation in Fungi will be found in Metzenberg (1972) for *Neurospora* and Clutterbuck and Cove (1973) for *Aspergillus.*

1. Genome associated proteins

One of the provocative claims made for certain fungal cells is that they have no histone associated with their DNA (Leighton *et al.*, 1971). However, other workers report the recovery of histone from the chromatin of budding yeast (Wintersberger *et al.*, 1973) and *Neurospora* (Hsiang and Cole, 1973) and for the time being it seems possible that the earlier reports are explained simply by the difficulties involved in isolating chromatin from fungal cells.

2. Positive control of fungal genes

The best known example of prokaryotic gene control, the lac operon of *E. coli*, works by negative control (see discussion in Chapter 2). This statement simply describes the fact that the lac operon is switched on normally and is only turned off by the binding of the specific repressor protein to the DNA of the operator gene. Induction is, therefore, de-repression and involves combination of an inducer molecule with the repressor molecule, thereby rendering it an ineffective repressor. In systems of negative control, therefore, deletion of the operator causes constitutive synthesis of the enzymes of the operon. Actually most, though probably not all, bacterial operons are under negative control (see Chapter 2).

Positive control, on the other hand, describes genes or operons which are normally switched off, without prior activity or presence of a repressor protein, and are only switched on by the binding of an inducer to the operator or promoter region.

The present state of knowledge of genetic control mechanisms in Fungi is that all the best understood gene regulatory systems are apparently under positive control and no really decisive evidence exists for the presence of negatively controlled genetic units in these cells. An interesting interpretation placed on this by Cove (personal communication) is that positively controlled genetic systems might well be more suited to what is probably the normal eukaryotic situation, i.e. where each structural gene might be sensitive to several quite different regulatory molecules, acting in parallel, and the particular combination of regulatory molecules affecting a gene at any one time would determine its expression.

One enzyme in *Aspergillus nidulans* gives some indications of being the product of a negatively controlled gene, but the evidence is far from complete. The example is xanthine dehydrogenase II (Scazzochio *et al.*, 1973). From experiments on this system, it has emerged that this enzyme is inducible by nicotinate in wild type *Aspergillus* strains and that nitrate reductase and nitrate are both necessary for effective repression of this enzyme. Mutants lacking the enzyme have been isolated and characterized and some appear to have lost their capacity for inducibility by nicotinate. This body of evidence does not add up to confirmation of an inducible enzyme under negative control, but such an explanation cannot currently be excluded.

Having discussed the one possible exception, the fungal genes which suggest positive control mechanisms will now briefly be discussed. First, it has been shown by Roberts (1970) that recessive mutations in the *gal A* locus of *Aspergillus* lead to loss of inducibility of two enzymes, galactokinase and galactose-1-phosphate uridyl transferase. Apparently the product of the *gal A* gene has, perhaps amongst other functions, that of positive induction of the genes for these two enzymes. Second, Cove (1969), Pateman and Cove (1967) and Cove (1970) have accumulated data from numerous experiments with strains of *Aspergillus* suggesting that at least six additional genes are likely to be under positive control. The genes are *nir A, amd R, ua Y, apl A, amd T* and *are A*. Various aspects of the accumulated data point to such a mechanism. For example, in the case of the *nir A* gene, the relative mutation frequencies of the wild type to *nir A* and *nir* A^c*-1* alleles have been determined. These alleles, when expressed in the mutant strain, give cells which give very low levels of nitrate and nitrite reductase, both in the presence and absence of nitrate (*nir* A^-) and constitutive synthesis of nitrate reductase and partly constitutive synthesis of nitrite reductase (*nir* A^c*-1*). From the data accumulated the mutation to *nir* A^- is much more frequent than the mutation to *nir* A^c*-1*. If gene control is negative, as with the lac operon of *E. coli*, then one expects that constitutive mutations, due to regulator gene alterations, will be much commoner than a mutation leading to non-induction, resulting from an impairment in binding of inducer and represser protein. In a positively controlled system, however, the reverse argument will apply and regulator gene mutations would lead to non-inducibility. Thus, the *nir A* evidence strongly favours a positively controlled gene.

Rather similar findings have been obtained for the other loci mentioned. For example the *ua Y* gene produce is necessary for the expression of at least three other genes, the *apl A* gene product is necessary for early steps in nicotinate degradation (Scazzochio, 1973) and the *are A* gene product is necessary for a number of enzymes involved in nitrogen metabolism (Arst and Cove, 1973).

Metzenberg describes a number of genes in *Neurospora* which behave as if under positively controlled regulations (Metzenberg, 1972).

3. Induction by product

An interesting phenomenon has come to light in work on certain fungal enzymes and that is apparent induction of a gene by an end product of the relevant metabolism, i.e. positive feed back. Such a system seems to have the facility to induce enzymes for product degradation once a high product concentration is reached, so it amounts to a procedure for rapid but not unlimited escalation of a particular metabolic pathway. Experiments which suggest such a mechanism in *Aspergillus* have been carried out by Scazzochio and Darlington (1968); Hirschberg and Ensign (1972) and Palleroni and Stanier (1964) have reported similar mechanisms in bacteria.

4. Gene clustering

The location of four genes in the budding yeast (*Saccharomyces cerevisiae*) on the fifth chromosome has been correlated with the time of expression of these genes and so linked to a linear reading mechanism for the control of transcription (see discussion in Section 1A). That example is one of genes clustered relative to the timing of transcription in the cell cycle. The other type of gene association on the chromosome one might expect is clustering of genes coding for enzymes which are involved in the same metabolic pathway, either acting in a fashion analogous to a bacterial operon with a common regulator and operator gene, or in some slightly similar way. First, it should be stated clearly that no example of an operon is known in a eukaryotic cell (i.e. two or more structural genes controlled by a single operator gene). But, nevertheless, there are a number of examples in fungal cells of genes coding for related proteins being clustered, and those we will now discuss.

At the outset of our discussion of such gene clustering, the difficulty of getting absolutely certain evidence for such a phenomenon should be stressed. One cellular situation which can look very like gene clustering is where a particular polypeptide has more than one enzymatic activity, and so may be recorded as two separate proteins which map together and are co-inducible. Other examples, and this accounts for many of the fungal cases, are closely related polypeptides which may aggregate under certain environmental conditions. Many such polypeptides may be examples of evolutionary divergence of originally identical genes, analogous to the close juxtaposition of the human β and δ globin genes. Such examples, although of interest in terms of genetical evolution, may tell us little about the control of eukaryotic gene expression. It should also be stressed that in fungi, as in other eukaryotic cells studied, genes concerned with successive steps in a metabolic pathway appear to be normally scattered through the chromosomes. This holds true for both *Neurosopora* and *Aspergillus*, in both of which, intensive genetic analysis has revealed independent assortment of such genes. The *arom* region of *Neurospora* illustrates some of the difficulties mentioned above, since a polypeptide aggregate is involved and probably only a single messenger RNA for the supposed gene cluster (Giles *et al*., 1967). It is still impossible to determine whether this is an example of a group of separate genes transcribed into a polycistronic message (which, in some definitions, would be an operon; the lac operon of *E. coli.* is transcribed into a polycistronic message) or a single polypeptide with multiple functions and an ability to aggregate in groups under certain circumstances.

The best example of true gene clustering of related genes, but excluding the possibility of transcription as an operon, comes from work by Cove and Pateman (1967) on nitrate reduction in *Aspergillus*. For discussion of examples in *Neurospora*, the reader is referred to the review of Metzenberg (1972) and in *Saccharomyces* to the reviews of Fink (1971) and Kuhn *et al.* (1972). Two genes *nia D* and *nü A* are both found to be located on the *Aspergillus* linkage group VIII and most of the available evidence points towards these genes being, respectively, the structural gene for nitrate reductase and for an enzyme catalysing the complete reduction of nitrate to ammonium. Analysis of 1770 progeny from *nia D* and *nü A* crosses gave a recombination rate of 2·6% (Cove, unpublished) which is higher than one would expect for completely adjacent genes but sufficiently low to suggest close linkage. No genes are known which map between these two loci. A third gene, *nir A*, produces a regulatory product affecting both the *nia D* and *nü A* loci, but present evidence reveals differences in the reaction of the two loci to changes in the regulatory gene locus which seem to argue against a coordinate transcription of *nir A* and *nü A* (Cove, unpublished). The regulator gene is loosely linked to the other structural gene loci. There are indications, from studies of the inducing effects of nitrate on these genes, that both positive and negative control mechanisms may be in operation, but the picture is not very clear at the present. What can be concluded is that *Aspergillus* affords an example of two structural genes, very closely linked in both chromosomal location and metabolic role in the cell, both powerfully affected by a regulator gene distant from them but still on the same chromosome. If the polypeptide chains coded by the two structural genes should turn out to be very different in amino acid sequence (thus arguing against recent gene duplication) then this phenomenon might be seen as a distintegrating bacterial type operon. If bacterial-type operons proved unsuited to the degree of genetic dexterity required from the genome of a eukaryote, then perhaps, in the course of evolution, the one-time operon groupings of prokaryotic genomes would drift apart and cease to be transcribed into polycistronic messenger molecules. Of course many other interpretations could be placed on the fungal data.

5. Comment

Probably the most striking conclusion which we can draw from this brief sketch of fungal gene control is that one has to scratch around very diligently to find anything resembling a prokaryotic operon. Gene clustering in other eukaryotic cells is an equally elusive topic. The genes for β and γ globins in man are closely linked and an operon has been suggested (see discussions in Whitehouse, 1973). An intensive scrutiny of *Drosophila* genes was carried out by Elston and Glassman (1967) in an attempt to correlate linkage with function. Their main conclusion was that functionally related genes were not significantly clustered, but that morphologically related genes were. That is, there is a tendency for genes affecting the wing or the eye to be linked on the same chromosome. No satisfactory explanation is known to account for this.

Fungal genetics is not simple and the metabolic pathways involved, especially those of intermediate metabolism, are obviously challenging and difficult to investigate. But because of their small genome, comparatively primitive evolutionary state and ease of bulk culture, they do seem to constitute a most important experimental inroad into an understanding of controlled gene expression in eukaryotes. Even if the conclusions cannot be applied without hesitation to all eukaryotes, their position as primitive eukaryotes is a particularly valuable one.

E. Nuclear transplantation and microinjection

The artificial transplantation of cell nuclei is an extremely powerful technique in cell biology and many of the old questions about nucleo cytoplasmic relations find answers in this situation. Whether the answers, now that we have them, are as satisfying as we expected them to be, is another matter. In many ways, it now appears that the initial questions were too simple.

Nuclei may be transplanted by any one of a number of different techniques involving micropipetting, other micromanipulative procedures or the formation of fused cells with the help of attenuated cellular viruses. These techniques are now very well known and have been exploited and reviewed by Gurdon (1974) and Harris (1974), respectively. Many interesting questions can be asked about nuclear transplantation experiments, particularly since they can be carried out in different ways. By prior enucleation of a cell, either by micromanipulation or ultraviolet irradiation, old cytoplasm can be combined with new nuclei. By combining nucleus and cytoplasm from different cell cycle stages of the same cell line, from differently differentiated tissues from

the same organism or even from the same cell type from different species, it has been possible to explore the signals exchanged by nucleus and cytoplasm in determining cell cycle development or tissue differentiation and determination.

On the other hand, heterokaryons may be formed using nuclei from different differentiated tissues of the same species or nuclei from widely different species and the effect of both nuclei and cytoplasm on one another observed.

The chief drawback to studies on gene expression which involve nuclear transplantation is that the molecules active in initiating or controlling changes cannot easily be identified. Essentially, the onus is on the choice of the experimental situation, the particular combination of nucleus and cytoplasm, to provide useful information. All the experimenter can normally do is stand back and observe survival and development of the new cell. This criticism must be somewhat modified, however, in that by the clever use of cell strains with particular metabolic requirements or of sensitive immunological assays, it has proved possible to identify certain proteins synthesized by transplanted nuclei or fused cells.

A technique in some ways related to nuclear transplantation involves the microinjection into cells of cytoplasm or cytoplasmic derivatives. This may involve the rescue of genetically defective or damaged cells by extraction of cytoplasm rich in DNA, the so called "germ plasm", or more recently the remarkable techniques of injecting specific messenger RNA molecules into cells which would not normally be expected to harbour such a species of messenger RNA.

1. Nuclear transfer by micromanipulation

Although the earliest experiments of this sort were carried out on amoebae (Commandon and de Fonbrune, 1939; Goldstein and Prescott, 1967), only the experiments involving amphibia will be discussed here. This is justifiable on the grounds that the yield of information has been greatest in the case of the amphibian experiments. Early success in transplanting a foreign nucleus was obtained with the frog *Rana pipiens* by Briggs and King (1952). These authors transplanted somatic cell nuclei into enucleated eggs and then allowed the embryos to develop. Most of the embryos were defective and these authors concluded that the somatic nuclei had lost their totipotency.

The more recent experiments of Gurdon (1962) and Laskey and Gurdon (1970) with *Xenopus* have yielded clearer results which will be discussed in greater detail. Two series of experiments have yielded the most decisive results here. In the first, nuclei were recovered from intestinal epithelial cells of feeding tadpoles and injected into *Xenopus* eggs whose nuclei had been destroyed by u.v. irradiation. Some apparently normal adults were reared from these implanted eggs (see Fig. 9). Experiments with nuclei from other differentiated cell sources were less successful and we should ask ourselves why the intestinal epithelial cell has proved the most satisfactory nuclear donor. Gurdon interprets the comparative success as a result of the rapid division of these cells. The possibility that the isolated nuclei were derived from only slightly differentiated cells must, therefore, be remembered, although Gurdon comments that many of the dissociated intestinal epithelial cells possessed brush borders (Gurdon, 1974).

For the second series of experiments by Laskey and Gurdon (1970), nuclei were obtained from adult *Xenopus* tissues such as skin, kidney and lung. In these experiments, if the nuclei were transplanted into eggs directly from the adult tissue, little development ensued (Gurdon and Laskey, 1970). If the donor tissue is explanted and allowed to grow on in culture prior to the nuclear transplantation procedure, then normal adults were obtained. Of course, when such explants are made and cell growth and multiplication occurs in tissue culture, it is crucial to ensure that the cell donating the nucleus is indeed truly differentiated and not the recent product of a stem cell division. It has, fortunately, proved possible to identify the donor cell as a differentiated one in the case of *Xenopus* skin since all the skin explant cells are found to possess keratin by direct visual examination and by challenge with fluorescent anti-keratin antibodies (Gurdon, 1974; Gurdon *et al.*, 1975). There is overwhelming statistical evidence that the actual nuclei yielding swimming tadpoles are from keratin-containing cells.

A further possible objection to nuclear transfer experiments of this sort is the accidental survival of the allegedly inactivated host cell nucleus (normally the egg nucleus). It has proved possible to overcome this objection by utilizing the mutant form of *Xenopus laevis 0-nu*, which lacks nucleoli (Elsdale *et al.*, 1958). Nuclei have been recovered from the *1-nu* cells of the hetrozygote, carrying with them the label of a single nucleolus. When such nuclei are used in nuclear transplantation experiments, as

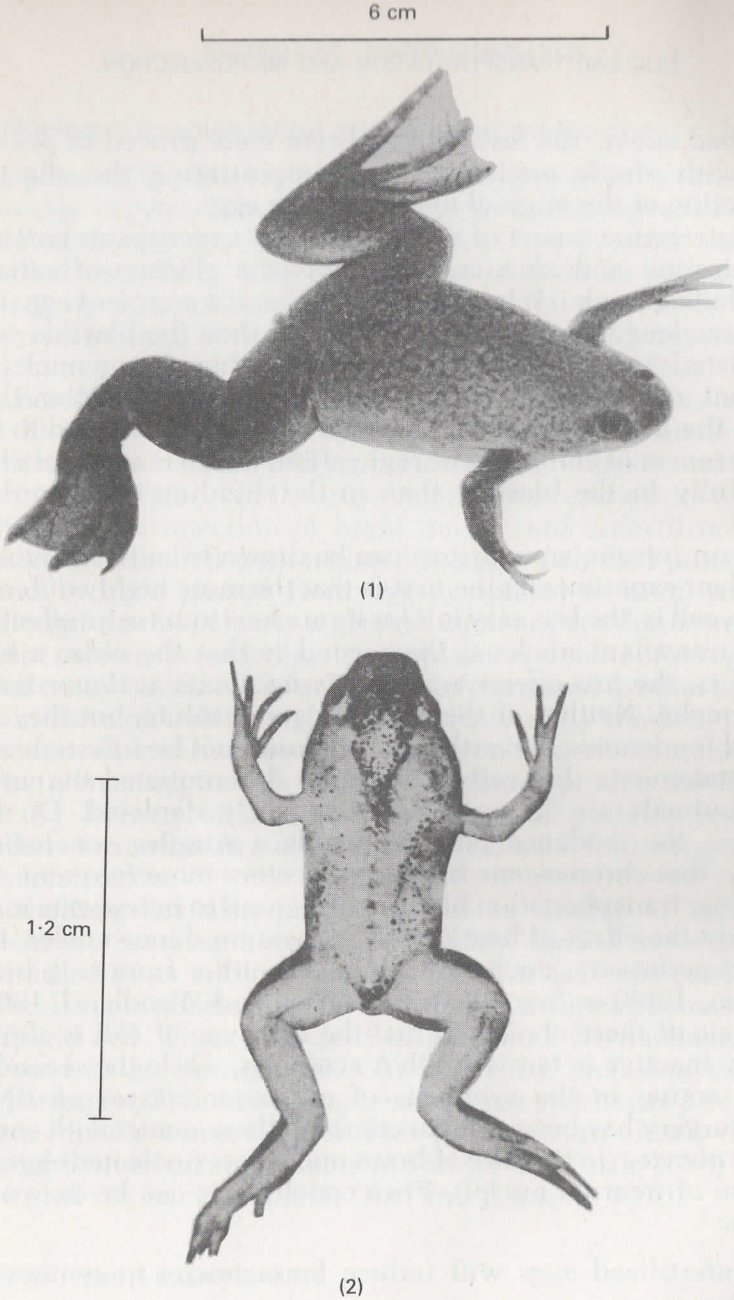


FIG. 9. Frogs produced by the transplantation of nuclei from cultured cells of *Xenopus laevis*. (a) A normal adult frog resulting from the serial transplantation of nuclei from cells which had been grown in culture for over a month. The cell culture was derived from a piece of epidermis of a swimming tadpole. (b) A recently metamorphosed frog which resulted from the serial transplantation of nuclei of cultured skin cells. The cultured cells were used as nuclear donors one week after they had grown out from a small piece of foot-web skin of an *adult* frog (from Gurdon, 1974).

described above, the resulting embryos were proved to possess cells with single nucleoli, thus demonstrating the effective destruction of the original nucleus of the egg.

An interesting aspect of these transplant experiments employing intestine and skin cell nuclei is the efficacy of serially transplanted nuclei. It has been found that if a recipient egg plus donor nucleus is grown on to a blastula, then the blastula cells dissociated and these nuclei used as further donor nuclei in recipient eggs, the resulting eggs will develop better than would the first transplant eggs. This seems to be related to the completeness of chromosome replication, which is accomplished more fully in the blastula than in the dividing egg (Gurdon, 1974).

Certain interim conclusions can be drawn from these nuclear transplant experiments; the first is that the more highly differentiated a cell is, the less easy is it for its nucleus to act as an effective donor transplant nucleus; the second is that the older a host animal is, the less effective are its tissue nuclei as donor transplant nuclei. Neither of these effects are absolute, but they are marked tendencies. Nevertheless, it should not be inferred from these statements that cells from more differentiated tissues or older animals are in any way genetically depleted. On the contrary, the evidence points towards a simpler conclusion, namely, that chromosome breakage becomes more frequent.

Nuclear transplantation has also been used to investigate more precisely the effect of host cell cytoplasm on donor nuclei. For these experiments, nuclei were derived either from frog brain (Gurdon, 1968) or frog neurula (Gurdon and Woodland, 1969). The logic of these choices is that the first type of cell is almost entirely inactive in terms of DNA synthesis, while the second is highly active in the synthesis of all major classes of RNA. Microsurgery has been used to combine these nuclei with entire eggs or oocytes (in the case of brain nuclei) or enucleated eggs (in the case of neurula nuclei). Firm conclusions can be drawn as follows:

1. Unfertilized eggs will induce brain nuclei to synthesize DNA.
2. Enucleated unfertilized egg cytoplasm will repress the normal RNA synthesis of an ex-neurula nucleus.
3. In general, the cytoplasmic milieu has the power to impose on a nucleus its own commitment, at least in terms of activity or inactivity of DNA or RNA synthesis.

2. Nuclear transplantation and labelled molecules

It is possible to push further along the line of inquiry into effects of nuclei on cytoplasm and vice versa, by including radioactively labelled nuclei in non-labelled cytoplasm or cold nuclei in labelled cytoplasm. The chief object of such an exercise is to follow by autoradiography the passage of previously tagged molecules in and out of nuclei and, hopefully, to determine their role in any changes which transplantation may induce. Thus it has been found that, if nuclei are transplanted into cells containing histone tagged with radioactive iodine, the histone will enter the new nucleus and accumulate in it (Gurdon and Woodland, 1970). Rather similar experiments were carried out by Arms (1968) by the injection of brain nuclei into unfertilized eggs, previously labelled with tritiated luecine. Labelled protein was found to have passed from egg cytoplasm to brain nucleus. Although there is clearly a general tendency for donor nuclei to accept proteins from recipient cytoplasm and some of this protein uptake may be selective for histone as compared to other proteins of similar molecular weight (Bonner, quoted by Gurdon, 1974), it is not possible to draw more weighty conclusions from the data.

An interesting, though slightly incidental, point is the observation by Byers (1963) of the movement of proteins from one nucleus to another in a multinucleate amoeba, following nuclear transplantation.

Labelling has also proved useful in studies of cell cycle synchrony in transplanted cells. With the careful use of tritiated thymidine as an autoradiographic label, it has been established that cell cycle phasing can be imposed on a donor nucleus by the recipient cell. Thus, a nucleus taken from a G1 nucleus can be induced to go through DNA synthesis by inclusion in a cell preparing for S, but a G2 nucleus will not synthesize DNA even when included in a G1 cell. This point is most clearly demonstrated by nuclear transplants in Stentor (De Terra, 1970) and by fusion of tissue culture cells (Rao and Johnson, 1970).

3. Cell fusion experiments

Cell fusion affords a useful experimental alternative to the transplantation of individual nuclei. Using a technique originally pioneered by Okada, many laboratories have recently made artificial cell fusions between cells of different tissues and species by exposing the cells to Sendai virus which had previously been

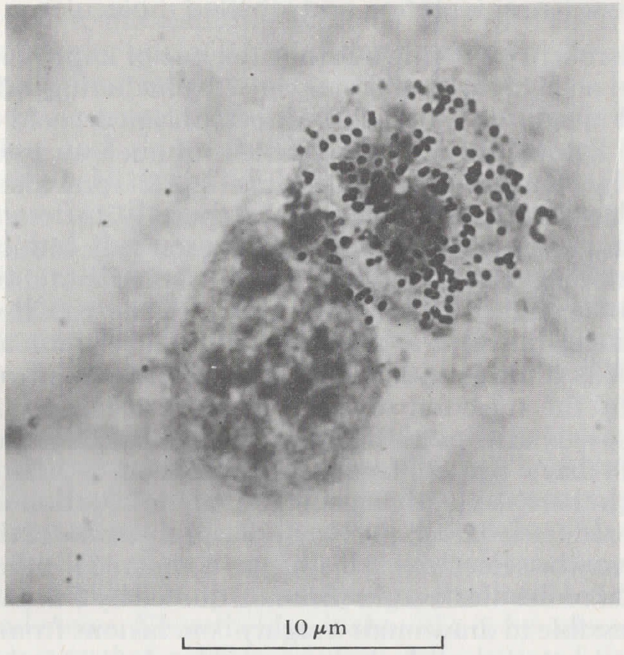


FIG. 10. An artificial heterokaryon formed by cell fusion. Autoradiograph of a binucleate cell containing one HeLa nucleus and one Ehrlich nucleus. The HeLa cells had been grown in (^{3}H) thymidine before the heterokaryons were produced. The HeLa nucleus is labelled and the Ehrlich nucleus is not (photo kindly provided by Professor H. Harris; from Harris, 1974).

inactivated by exposure to ultraviolet light. The Sendai virus has the property of making the cells sticky and inducing fusion between neighbouring cells, while the attenuation of the virus ensures no further viral attack on the cells. The resultant multinucleate fused cells can be secured in various combinations, those having nuclei of differing types being termed heterokaryons (see Fig. 10). A further extension of the cell fusion technique involves the ability of the virus to lyse nucleated erythrocytes, so permitting the incorporation of erythrocyte nuclei in heterokaryons almost entirely devoid of erythrocyte cytoplasm. A review of experiments involving cell fusion will be found in Harris (1970a).

As with the nuclear transplantation experiments, these cell-fusion heterokaryons demonstrate the relative plasticity of the

differentiated nucleus, in that it is able to respond to the influence of the cell into which it becomes incorporated. By combining cells of differing characteristics together, it can be discovered that metabolic activity is normally dominant over metabolic inactivity. Rabbit macrophages normally make RNA but no DNA, hen erythrocytes make neither (see below) and Hela cells make both. Combinations of these cells demonstrate that both RNA synthesis and DNA synthesis can be induced in nuclei normally inactive in either.

Perhaps the most significant heterokaryons made by Harris are those of hen erythrocyte nuclei in combination with rabbit macrophages, mouse fibroblasts or Hela cells. Two hen specific proteins are found to be synthesized by these fused cells, the one a surface antigen detected by an immunological assay and the other a soluble enzyme, inosinic acid pyrophosphorylase, which has the job of catalysing the condensation of hypoxanthine with ribosyl phosphate, prior to its incorporation into nucleic acid. A fortuitous mouse mutant, which lacks this enzyme, was used as a source of mouse fibroblasts (Harris and Cook, 1969) and, therefore, the appearance of the enzyme in the heterokaryons was clearly due to the activity of genes within the chicken erythrocyte nucleus. Moreover, neither of these proteins appeared until after development of a nucleolus within the hen erythrocyte nucleus, implicating this organelle in the chain of events leading to the synthesis of these proteins.

One unfortunate aspect of the Harris experiment was the assumption that the hen erythrocyte was entirely inactive in RNA synthesis. Such a notion stems from the work of Cameron and Prescott (1964), but is, in my experience, quite untrue. Chicken erythrocytes normally synthesize a substantial amount of RNA (Madgewick, 1972; Maclean and Madgewick, 1973; see Fig. 11). The initial absence of RNA synthesis on the part of the chicken erythrocyte nuclei in Harris' experiments is, presumably, a temporary result of the cell lysis and fusion procedure. Nevertheless, these experiments give strong grounds for believing that considerable shifts of genetic commitment and nucleic acid synthesis are possible, even in nuclei from differentiated cells, provided, at least, that such nuclei are within the G1 phase of the cell cycle.

It is worth mentioning here that Harris' laboratory has now proceeded to employ the technique of cell fusion to combine malignant and non-malignant cells. Malignant cells, in

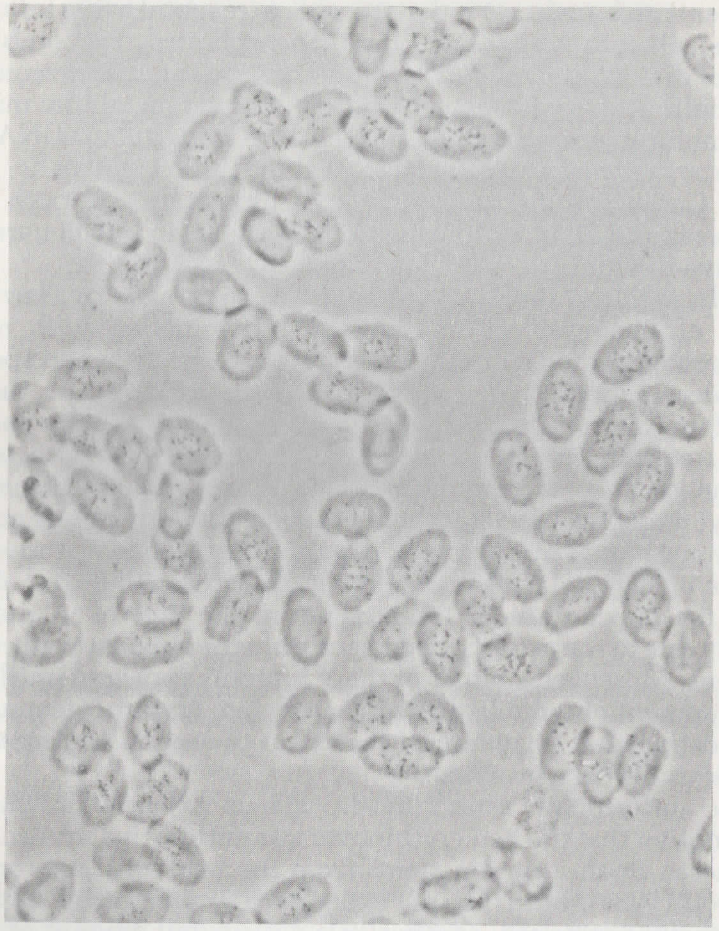


FIG. 11. Autoradiograph of erythrocytes from a mature adult chicken following incubation of the cells for four h in a culture medium containing tritiated uridine. Notice that most of the cells contain nuclei which have been active in RNA synthesis during the culture period. Cells are fixed and TCA extracted after fixation.

combination with poorly malignant cells, lose much of their malignant character (Weiner *et al.*, 1973) and indeed fused combinations of two separate highly malignant cell strains may also be poorly malignant. Moreover, cells of low malignancy such as A9 mouse cells, seem to owe much of their power to suppress

malignancy in the heterokaryon situation to the possession of certain chromosomes. If certain specific chromosomes are lost from the heterokaryons, the malignant character may once more be displayed (Allerdice *et al.*, 1973).

4. Microinjection of cell contents other than nuclei

The microinjection technique permits the transport of cytoplasm from a donor cell into a recipient cell and, indeed, such a technique is often easier than the transplantation of intact nuclei. One of the major problems with cytoplasmic transplantation is that of designing a meaningful experiment, since any added cytoplasm is, of necessity, diluted by the cytoplasm of the recipient cell. The most useful experiments employing this technique are designed as rescue experiments in which a cell, already defective and destined to betray its defects in later abnormalities or death, may be given a dose of foreign cytoplasm which then has the power to ensure a normal development for the egg. Such experiments have been undertaken by Smith (1966) in which fertilized frog's eggs were irradiated with u.v. at the vegetal pole, thus destroying the germ plasm. Such eggs give rise to tadpoles which lack germ cells. However, when Smith injected vegetal pole cytoplasm from a normal fertilized egg into the irradiated eggs, a proportion (less than 50%) gave rise to tadpoles which possessed germ cells. Since u.v. light at 260 mm is most effective in destroying the endogenous germ plasm, it is likely that both the original germ plasm and that carried over by the micropipette contains essential RNA. Unfortunately, it is not possible to be more definite about the identity of the active donor molecules.

A slightly different type of rescue experiment has been devised by Briggs (1969) in which use was made of a maternally inherited genetic defect in the American oxolotyl *Ambystoma mexicanum*. The defect is that eggs of female axolotyls, homozygous for the gene o (ova deficient), are arrested early in development after fertilization. Briggs has found that such eggs, if injected with cytoplasm from a normal wild-type egg, may grow normally. Two other aspects of this experiment heighten its interest. First, defective eggs cannot be rescued by injection of a wild type blastula nucleus and, second, the rescue material, which appears to be concentrated in the normal oocyte nucleus, is trypsin sensitive and, therefore, perhaps proteinaceous in character.

Such rescue experiments, although perhaps not greatly informative in themselves, do underline the potential use of microinjection of cytoplasm, especially of selected cytoplasmic fractions or components.

5. Microinjection of messenger RNA

An exciting possibility opened up with the availability of relatively pure preparations of messenger RNA, namely that of injecting messenger RNA into living cells. It was a particularly attractive proposition to inject into a cell message for a protein which would not normally be synthesized in such a cell, or even, in the best cases, a cell which did not possess a gene coding for such a protein. Such experiments might be expected, at the least, to yield information about the universality of the code and the general applicability of the concept of messenger RNA and, at best, to be informative about the half-life of mRMA, translational control, ribosomal specificity and other questions.

Messenger RNA has, of course, been successfully used in cell free systems (see review by Lingrel, 1974). Such work has chiefly underlined the considerable, though not absolute, dependence of messenger RNA translation on specific initiation factors, often apparently relatively cell-specific. Other uses of messenger RNA have involved exposure of tissue cells to external applications of messenger RNA, followed by a search for morphological changes consequent on the hoped-for uptake of some intact mRNA. Some early experiments of this type were undertaken by Niu and his co-workers (Niu *et al.*, 1961), in which chick blastoderm cells were exposed to chick heart RNA and then scored for the appearance of pulsating myoblasts normally characteristic of the tissue donating the RNA. A great deal of controversy has surrounded these and other similar experiments, especially because the specificity of added message has not been correlated with the appearance of the protein for which it codes. A recent review of these experiments will be found in Lane and Knowland (1974).

The credit must go to Gurdon *et al.* (1971) and Lane *et al.* (1971) for the primary work of actually injecting into living cells, via a microneedle, preparations of messenger RNA. Rabbit, mouse and duck globin mRNA have been most frequently used, the RNA being injected into unfertilized *Xenopus* oocytes still retained within an oocyte cluster together with the follicle cells. Since a radioactive label was necessary for the detection of newly synthesized protein, and little or no amino acid uptake is practised by such oocytes, the label was also applied by microinjection. Using a variety of methods, it was demonstrated by Gurdon and his colleagues that the globin mRNA was translated into globin and also that translation accurately reflected, in the protein synthesized in the oocyte, the particular globin which was characteristic of the species donating the mRNA.

The importance of Gurdon's experiments can scarcely be exaggerated, demonstrating as they do the accurate translation of a foreign messenger RNA following its artificial and, at a cellular level, its gross insertion into the cellular environment.

It is appropriate to take time to outline here exactly what these experiments have demonstrated. A full discussion will be found in Gurdon's own account of his work (Gurdon, 1974). First, there is impressive evidence for the fidelity of translation of injected messenger RNA not only for globin mRNA (see Table 2), but also for mRNA for mouse collagen, EMC virus, calf lens crystallin, trout testis protamine, mouse myeloma light chain and honeybee promellitin (Gurdon, 1974). There is additional evidence that some mRNAs are not effectively translated, however, including RNA phages (Lane and Knowland, 1974) and certain tumour viruses. In addition, with the mRNA preparations which are translated, not all are translated with the same efficiency. Gurdon has calculated that the mouse and rabbit globin mRNA is translated at an efficiency which falls within the normal cellular range, i.e. each functional β globin mRNA molecule yields about 30 globin molecules/min. But, interestingly, the relative rates of translation of the α and β globin mRNAs are different in the oocyte and this variation also differs from the variation in translation rate, which these mRNAs display in the reticulocyte. In other words, the oocyte and the reticulocyte process the same mRNA molecules slightly differently in the process of translation. Gurdon attributes the effect, at least in part, to an influence of haemin on translational rates.

Message injection into *Xenopus* oocytes has also given clear support to the idea of a cell having some spare translational capacity and, therefore, to the effectiveness of mRNA as a limiting factor in protein synthesis. This evidence emerges from experiments in which varying amounts of message were injected into oocytes up to saturation levels. First, when message was added in low doses, the endogenous message continued to be translated at the same rate, demonstrating that extra ribosomes and transla-

TABLE 2. Characteristics of translation of globin messenger RNA injected into frog oocytes (from Gurdon, 1974).

Species of Hb mRNA injected	Fractionation method	Result of fractionation (Oocytes labelled with ^{3}H-his; carrier Hb labelled with ^{14}C-his, or recognized by optical density (OD)).	Similarities between oocyte-derived and carrier Hb
Rabbit, duck and mouse	Sephadex G-100 in tris-glycine buffer	Retarded ^{3}H-peak coincident with OD of carrier Hb	Overall size of Hb
Rabbit	Polyacrylamide gel electrophoresis in tris-glycine buffer	Sharp ^{3}H-peak coincident with OD of carrier Hb	Overall size and charge of Hb
Rabbit, mouse and duck	CM cellulose chromatography of free globin chains; (elution conditions will separate globins of rabbit, mouse, duck and frog.)	Coincidence between all globins of donor species and oocyte-derived materials.	Overall charge of each major globin species
Rabbit	Gradient elution of tryptic peptides from cation exchange resin	Coincident elution, and similar specific activity, of ^{3}H-oocyte-derived and ^{14}C-carrier peptides (for 7α and 8β globin peptides)	General similarity in charge for about half of all tryptic peptides in each kind of globin
Rabbit and duck	Paper electrophoresis at pH 3·5 and pH 6·5, and paper chromatography.	As above for 7 peptides from both α and β rabbit globins, and for 5 or 6 peptides of each major duck globin.	

* Details of these results may be found in Lane *et al.* (1971), Marbaix and Lane (1972), Lane *et al.* (1973) and Gurdon *et al.* (1974).

tional enzymes were available in the oocyte over and above those committed to the current translation of endogenous messenger RNA (Moar *et al.*, 1971). Second, the saturation value for added message comes in at about 30 × 10^9 molecules, at which value the added message is accounting for some 30–50% increase over the protein normally synthesized. What is most intriguing about this system is that, above the saturation value for added message, little more globin is synthesized but a minimal decrease in endogenous protein synthesis ensues. In other words, there is not open competition for translation between added message and endogenous message. It is still not clear whether message and/or ribosomal specificity is involved here, or simply a preference for messages already in use, although the evidence of Lingrel and Woodland (1974) and Gurdon (1974) favours the interpretation of an unspecific message-activation molecule which would, on combination with mRNA, ensure its regular translation, but without which the mRNA would remain idle and might be destroyed. With such an explanation, addition of added globin mRNA becomes saturating when it has mopped up all of the available, and currently unused, message activator.

On the questions of message stability, the message injection experiments also reveal some interesting facts. First, there is no detectable message turnover during the life of the oocyte following message injection. Up to the two week period of oocyte life in culture, translation of the added message continues at a rate commensurate with negligible mRNA turnover. Now it could be argued that oocytes are highly abnormal cells and that message turnover would be much more likely in a dividing cell situation. Such an example is afforded by the growing tadpole. Apparently, such an experiment is less easy than oocyte injection since the fertilized eggs are much more sensitive to the micro-injection procedure than is the oocyte. Abnormal cleavage frequently follows microinjection and the injected material is apt to be relegated to the uncleaved portion of the embryo (Brachet *et al.*, 1973). These difficulties have now been largely overcome and some feeding tadpoles obtained from message injected fertilized *Xenopus* eggs (Gurdon, 1974 and Gurdon *et al.*, 1974). Mouse and rabbit globin synthesis could still be detected at a substantial level, in the feeding tadpoles, indicating that the mRNA was as stable in the dividing cell situation as had been recorded for the oocyte. This at least indicates that injected message is not selectively broken down or discarded. In addition, Gurdon has analysed the anatomical location of the added globin mRNA in the tadpole and finds that, as measured by production of the appropriate globin, it is generally distributed and certainly not restricted to sink cells or tissues normally synthesizing endogenous globin. In other words, there seems to be no mechanism here for selectively destroying or inactivating unwanted messenger RNA.

The final step in the control of gene expression is the regulated stability of the ultimate product. In the case of proteins, mechanisms are widely known which will specifically store or destroy particular molecular species, so determining their availability in the cell and the organism. Alternatively, proteins may be modified by a cell, either to accomplish a different task or as a step towards their inactivation or destruction. At this level too, something can be learnt from the message injection experiment by assaying the persistence of the foreign protein which comes to be synthesized in response to the added mRNA. Suitable experiments have not yet been carried out which might throw light on the first part of the question—which proteins are broken down and where? Since the mechanisms for controlled protein degradation might be expected to occur only in cells which would normally synthesize such proteins, the question demands examination of, say, mouse globin survival in the *Xenopus* erythroid cells, following mouse globin mRNA injection into fertile *Xenopus* eggs. The second part of the questions, that relating to the subsequent modification of proteins following their synthesis, has been examined in the message injection situation. For all proteins so far examined, it appears that their normal processing occurs even in the oocyte, despite the fact that they would not normally occur in that cellular situation (see comparative table in Gurdon, 1974). This suggests, although other explanations are possible, that the enzymes responsible for protein modification, are widely or universally distributed and may be common enzymes carrying out multiple tasks.

6. Comment

The experiments discussed in this section, involving manipulated living cells, either with different combinations of nuclei and cytoplasm, fused cells or simply added cytoplasm, have certain inherent advantages over many other systems. The experiments are conducted in the living cell and so worries about non-physiological effects are avoided. So too are the problems of applying to the living situation information gleaned from test-tube biochemistry. The disadvantages are that microinjection is slow and difficult and is easiest with oocytes and eggs. Cell fusion overcomes this difficulty but produces somewhat complex cellular situations. Probably the greatest disadvantage of these systems is the difficulty of identifying or characterizing the active molecules. Dramatic effects on subsequent cellular development may follow from the implantation of a foreign nucleus, but it is quite another matter to determine what molecules are particularly involved. Attempts to identify specific molecules synthesized in heterokaryons are encouraging but the interpretation of their significance is not without difficulties.

Many of these shortcomings are overcome by the direct injection of specific RNA molecules into cells and these experiments must be regarded as amongst the most useful and promising of all those reviewed in this book. Not only does message injection provide supportive evidence about the nature of messenger RNA and the fidelity of its translation, but this work has also given the shape of a whole host of other pieces of the developmental jigsaw: messenger half life, messenger competition, messenger breakdown, protein processing and so on. There could be many more successes, of which some possibilities are now given.

1. Assay of exogenous protein half life in cells normally possessing such a protein.
2. Extension of the mRNA survival curve by following the maturation of animals derived from message injected eggs.
3. Curing of genetical defects by injection into defective eggs of mRNA for the non-defective form of the appropriate protein involved in the lesion.
4. Since mRNA must normally have a life span considerably shorter than that of the organism in which it occurs, microinjection of DNA genes, made by reverse transcriptase from the appropriate mRNA, seems a logical extension. This should promise perhaps indefinite persistence of the added information.

F. Cartilage

The differentiation of cartilage has been studied by several groups of workers, the early work depending on recognition of cartilage by its morphological characteristics. Increased knowledge of the biochemistry of this tissue has permitted the use of more refined criteria. In particular, an understanding of the synthesis of chondroitin sulphate proteoglycan and the biosynthetic pathway involved, together with the discovery of a unique cartilage collagen, have made the recognition of differentiating cartilage cells easier and more reliable. Cartilage can, therefore, take its place along with silk gland, mammary gland and other cell systems as a tissue which synthesizes and exports, at least outside the cell, some genetically determined products unique to itself. Recent discussions of cartilage development and differentiation will be found in Searls (1973) and Levitt and Dorfman (1974).

It is well to outline the characteristics of the molecules unique to cartilage and how they may be monitored. Chondroitin sulphates are mucopolysaccharides found in bone, tendons, heart valves, cornea and cartilage. They are biochemically termed glycosaminoglycans and when conjugated with protein, as they are in cartilage, are styled proteoglycans. The main glycans of cartilage are chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate, their relative quantities varying for cartilage of different ages, locations and species. In the structure of a proteoglycan, the polysaccharides exist as side chains attached to serine or threonine residues of a long polypeptide chain. Unfortunately, the identity and nature of the protein core of cartilage is not well understood, but it is of the order of 2000 amino acids long and may have repeating units (Mathews, 1971). For our purposes the main interest in the proteoglycan is that there is evidence that the protein core is a molecule unique to cartilage (Levitt and Dorfman, 1974).

The second molecule which is an important structural component of cartilage and is of limited tissue distribution, is collagen. This is a large and important structural molecule and is now known to be made up of fundamental units of tropocollagen, each of which is a triple helix of three polypeptide chains of about 1000 amino acids each. Different tropocollagen chains are to be found in differing tissues, almost certainly coded for by different genes. In current nomenclature (see Levitt and Dorfman, 1974), individual protocollagen chains are termed α chains, two cross-linked chains as β and three cross-linked chains as γ protocollagen. The basic chains were originally styled $\alpha1$ and $\alpha2$ but, unfortunately, it is now clear that a variety of $\alpha1$ chains exist.

Thus the necessity for the terminology of $\alpha1$ (i), $\alpha1$(ii) and $\alpha1$(iii) to denote the distinct forms of the $\alpha1$ chain. The tropocollagen arrangement of calf skin collagen, for example, is $(\alpha1\ (\mathrm{i}))_2\alpha2$, yielding the triple helix of the functional molecule. Chick cartilage, on the other hand, is $(\alpha1\ (\mathrm{ii}))_3$ and it now appears that this protocollagen is unique to cartilage (Levitt and Dorfman, 1974).

The present situation is, then, that biochemical markers exist for cartilage, but the detection of these markers may not be easy since they closely resembled molecules found in other tissues. Thus, although the uptake of sulphur may be an effective marker for the glycans, they are not unique to cartilage, occurring also in bone, tendon, cornea and elsewhere. However, cartilage differentiation *in vitro* does permit the uptake of sulphur to be employed as an indication of the onset of glycan synthesis and assembly. Mechanisms for the export of proteoglycan and collagen from the chondrocytes to the extracellular matrix of cartilage remains obscure.

1. Differentiation of cartilage *in vitro*

Numerous studies have centred on the culture of chondrocytes. These cells are not always easy to grow in culture, perhaps partly because they are often overgrown by non-cartilage cells present in the original explant. Very satisfactory cultures of chondrocytes are used in the Strangeway's Laboratory, Cambridge (Fell, personal communication) and are also reported by Levenson (1969). The term differentiation has often been applied to the apparent cessation of collagen and proteoglycan synthesis in explants of cartilage, the assumption being that when the chondrocytes were stimulated to divide by explanation into culture, this was incompatible with chondrocyte function and they ceased to synthesize their characteristic proteins (Holtzer and Abbott, 1968). These authors report that often, when chondrocytes are permitted to undergo prolonged growth in culture as monodisperse cell sheets, for periods of two weeks or more, the chondrocyte morphology is lost, as well as the ability to incorporate high levels of $^{35}SO_4$. If the altered cells are now permitted to grow in clonal conditions (seeded at a certain critical cell density into a special medium), only a percentage of the altered cells regain the chondrogenic function and produce clones of chondrocytes which synthesize matrices. These experiments clearly suggest that some sort of dedifferentiation process has occurred, by which only some cells are able to remember their determined programme of gene expression. Although there is still some difficulty in establishing cartilage in culture and particularly in preventing a rapid multiplication of chondrocytes or other cells (both of which seems to be at the expense of synthesis of the characteristic protein of cartilage), acceptable results have now been obtained, at least, in detecting synthesis of characteristic proteoglycan (reviewed by Levitt and Dorfman, 1974).

Of greater interest to use here are studies on the differentiation of chondrocytes in culture from non-cartilage primordia. Such studies were reviewed in 1967 by Thorp and Dorfman. The main evidence pointed towards a specific inducer molecule being secreted by notochord and ventral spinal cord, which would promote cartilage formation in the mesenchyme cells of explanted embryonic somites. In most instances, cartilage was identified by the cellular morphology of supposed chondrocytes and incorporation of $^{35}SO_4$ into substances which electrophoresed with chondroitin sulphate. More recent studies (Ellison and Lash, 1971) have stressed the role of culture conditions in chondrocyte differentiation *in vitro* and cast some doubt on the specificity of the alleged inducing molecules acting on the somite mesenchyme. Other observations of Lash (1968) also have a bearing on the subject of the induction of cartilage in embryonic tissue cultured *in vitro*. The work involved a careful search for cartilage-like chondroitin sulphate in pre-induced embryonic tissues, and, particularly, in the somite tissue from which chondrocytes may differentiate. Lash reports that embryonic somite can synthesize these molecules prior to induction, but only in very small quantities. He, therefore, draws a distinction between synthesis of small quantities of a tissue specific protein and the stabilization and enhancement of this production. The former state he refers to as "pre-induction" and the latter as "induction". Whether the embryonic somite cells are already determined to become chondrocytes when chondroitin sulphate synthesis mences, or whether this molecule is actually made by a wider range of related tissues, at least in embryo, cannot at the moment be answered. What is of note is the striking similarity between the findings of Lash (1968) in the differentiation of cartilage and of Rutter *et al.* (1968a,b) in pancreas development (the latter is discussed in the chapter on the pancreas). In both systems, a low level of production of the key protein commences and proceeds for some time prior to the large scale synthesis associated with overt differentiation.

2. Cartilage differentiation *in vivo* and in organ transplants

The differentiation of cartilage in early development has been studied and reviewed by Searls (1973). Many studies have also concentrated on tissue extracts and other substances which have the property of inhibiting cartilage differentiation *in vivo* (Toole *et al.*, 1972). Others have involved the use of teratogens, such as 3-acetyl pyridine (3AP), to induce chondrogenic expression in limb primordial mesoderm (Caplan, 1972). An interesting group of papers have followed from studies on cartilage differentiation in the postembryonic period, showing that extracts of bone have the facility to induce cartilage formation when injected into muscle (Reddi and Huggins, 1972 and Urist and Strates, 1971). As will be stressed in the final comment on this chapter, however, studies on cartilage differentiation are still at an early stage and the biochemical markers are difficult to utilize with precision. At the moment, cartilage differentiation has perhaps rather little to teach us about gene expression.

3. The effects of BUdR on cartilage differentiation

The use of 5-Bromo-2′-deoxyuridine (BUdR) is discussed more fully in Chapter 7, but we can briefly examine here the evidence emerging from its use on chondrocytes and their precursors. First, exposure of limb bud cells to low concentrations of BUdR during the period of determination may entirely prevent cartilage formation. The main mechanism for this effect seems to be a block in the formation of the two key proteins of cartilage: the protein core of the proteoglycan and the cartilage type collagen $(\alpha 1\ (ii))_3$. Interestingly enough, the progeny of BUdR treated cells continue to synthesize collagen of the $(\alpha 1\ (i))_2\alpha 2$ type while lacking the $(\alpha\ (ii))_3$ type, which strongly suggests that synthesis of the two types of collagen are under separate genetic controls (Levitt and Dorfman, 1974). Normally, when limb bud cells, previously grown over agar, are subcultured on to plastic dishes, very little flattening or spreading of the cells occurs, but limb bud cells previously treated with BUdR do spread and flatten in a characteristic manner (Silagi and Bruce, 1970). Suggestions that BUdR affects and alters the properties of cell membranes have also emerged from work on other types of cells. Although thymidine can be used to competitively prevent the effects of this drug, it will only do so in the chick limb bud cells when provided along with the drug. Provision of excess thymidine after the application of BUdR does not reduce its effects.

Cultured chondrocytes react to BUdR slightly differently from the embryonic cells. Production of a cartilaginous matrix is greatly reduced following exposure of cultured chondrocytes to the drug (Coleman *et al.*, 1970) and, as with the embryonic cells, flattening and spreading of the cells is evident. When chondrocytes have been established in culture for some days, however, exposure to BUdR has little or no effect. Similarly, if cartilage cells are organ-cultured in groups or incubated in the presence of inhibition of DNA synthesis during treatment, BUdR shows no effect on the cells. The explanation for the lack of effect of this drug on organ cultured cells seems to be tied up with cell division, since cells which are dividing rapidly in culture are more profoundly affected (Mayne *et al.*, 1973).

The effects of BUdR on both embryonic chondrocyte precursor cells and on differentiated chondrocytes in culture are in line with effects of this compound observed in other cellular systems. One result from the cartilage experiments seems to be exceptional, however. It is that, in some cases, where differentiated chondrocytes were treated, inhibition of synthesis of the proteoglycan core protein is transient if the drug is removed, (Levitt and Dorfman, 1974). Since these authors also observe an extremely rapid turnover and excision of DNA containing BUdR in limb bud cells, they suggest that this may account for the apparent recovery from the drug. In essence, then, the main findings of BUdR treatment of chondrocytes and chondrocyte precursor cells are that the differentiated characteristics of the cells are diminished or abolished and that, in the case of the primordial differentiating cells, the effects are invariably permanent. With the already differentiated chondrocyte, however, the effects of BUdR are very dependent on the rate of growth of the cells and, even when effective, the changes are sometimes reversible.

The effects of BUdR on cartilage cells will be discussed further in Section 7A, but it is appropriate to underline here the interesting pattern of effect which the drug displays. Its action shows remarkable selection for particular parameters associated with the differentiated state. Thus, with chondrocytes, it is the molecules particularly synthesized by these cells and by no others, which are affected. In the case of collagen, the more ubiquitous forms continue to be synthesized. Only the chondrocyte specific $(\alpha 1\,(\text{ii}))_3$ form is noticeably affected, as with the proteoglycan. Although some of the enzymes involved in the proteoglycan synthesis were present in reduced amounts in the treated cells, the effect was small compared to the great reduction in the manufacture of the chondroitin sulphate chains. It, therefore, seems most probable that BUdR particularly affects production of the proteoglycan core protein.

As discussed by Rutter *et al.* (1973) and by Levitt and Dorfman (1974), the picture which emerges from experiments involving exposure of cells and tissues to BUdR is that the most pronounced effects involve the more precise aspects of differentiation and the molecules whose synthesis is most greatly reduced are those specific to that cell or tissue. It is as if the substance had a preference for genes making specialized proteins, the "luxury molecules", to use the terminology of Holtzer and Abbott (1968).

4. Comment

In terms of increasing knowledge of how gene expression is controlled, cartilage is not a system in the front rank. Certainly, it takes its place along with a dozen or so other tissues as a type of cell with unique proteins of its own. But unlike, say, erythrocytes or lens cells, the proteins are not well characterized and are relatively difficult to distinguish from other somewhat similar proteins which are known to have much wider tissue distributions. Indeed, the evidence that the protein core of cartilage proteoglycan and the characteristic collagen of cartilage $(\alpha 1\,(\text{ii}))_3$ are specific to cartilage remains more of an assumption than a fact. However, the very fact that chondrocytes may synthesize proteins which are very similar but not identical to those found in other cell types is, if it is true, in itself of interest in terms of gene evolution.

Two other aspects of chondrocyte differentiation impinge on our interest in gene expression. The first is that of the apparent instability of chondrocyte determination, the ability of some such cells, when in culture and especially after prolonged rapid division, to lose or forget their original commitment. This assertion is of considerable interest and deserves to be made watertight. Examples of committed cells which lose or change their commitment are scarce in biology and they clearly are a potential source of information on how a genetic programme is normally fixed. The other aspect, which is also related to the alleged dedifferentiation, is the behaviour of chondrocytes and chondrocyte precursor cells, following treatment with BUdR. Here, the loss of differentiated phenotype and characteristic proteins is in line with other systems, but what is unusual is the apparent reversibility of the BUdR effect in some of the differentiated chondrocytes. Both observations, if taken together, suggest that the genetic programme is less stable in the chondrocyte than in many other cells. If the cells are grown rapidly in culture, the details of the programme may be lost and if the programme is selectively repressed by BUdR, it may sometimes be recovered.

G. Liver

Whereas most of the experimental systems discussed in this book are remarkable because they involve commitment of cells or tissues to the production of one or two specialized proteins, liver cells are noteworthy because they provide information on the coordination of production of very many different proteins, both during development and in adult life. Discussion of the regulated synthesis of the liver proteins will be found in an excellent review by Greengard (1971) and, in briefer form, in Truman (1974). The production of yolk protein by liver cells, following oestrogen induction, is discussed in Section 3.H.

In embryonic life, the mammalian liver arises as an outgrowth of the gut and numerous cell types are found within it. These include Kupffer cells, which are apparently fixed macrophages, and many other endothelial cells which go to form the walls of hepatic blood vessels and bile canaliculi. But the bulk of the liver is composed of lobules of cells, fairly uniform in appearance; these are the characteristic parenchymal cells of the liver, often termed hepatocytes. The mitotic index of the adult liver is very low, but is high in embryonic or regenerating liver. Liver functions as both an exocrine gland, secreting bile into the gut, and as an endocrine gland, secreting a variety of substances directly into the hepatic blood vessels.

If liver enzymes and other proteins are assayed during development of the organism, the times of appearance of synthesis are found to be highly variable. The variation is far from random, however, and Greengard (1971) has highlighted the interesting observation that three critical times of development can be recognized. At these times, many different proteins either appear for the first time or have radical changes in their rate of synthesis. Greengard's data is reproduced in Table 3. These times of critical change are not necessarily indicated by significant rises in the total protein content of the liver, but are indicated rather by sudden alterations in the production of some ten to twenty proteins.

TABLE 3. Enzymes accumulating in rat liver during three developmental stages. Biphasic enzymes are printed in italics: the numbers printed in columns under E.C. are the identifying code for that enzyme in the classification of the Enzyme Commission (from Greengard, 1971).

Late foetal cluster	EC	Neonatal cluster	EC	Late suckling cluster	EC
UDPG glycogen glucosyltransferase	2.4.1.11	Phosphoenolpyruvate carboxylase	4.1.1.32	Glucokinase	2.7.1.2
UDPG pyrophosphorylase	2.7.7.9	*Glucose 6-phosphatase*	3.1.3.9	Ornithine aminotransferase	2.6.1.13
Phosphoglucomutase	2.7.5.1	Histidine ammonia-lyase	4.3.1.3	Tryptophan oxygenase	1.13.1.12
Glycogen phosphorylase	2.4.1.1	Tyrosine aminotransferase	2.6.1.5	Alanine aminotransferase	2.6.1.2
Glucose 6-phosphatase	3.1.3.9	L-Serine dehydratase	4.2.1.13	*Asparaginase*	3.5.1.1
Carbamoylphosphate synthetase	2.7.2.5	Alanine glyoxalate aminotransferase	None	*Arginase*	3.5.3.1
				Glutamine synthetase	6.3.1.2
Ornithine carbamoyltransferase	2.1.3.3	*Asparaginase*	3.5.1.1	Malate dehydrogenase (NADP)	1.1.1.40
Argininosuccinate synthetase	(6.3.4.5)	5′-Nucleotidase	3.1.3.5	ATP citrate lyase	4.1.3.8
Argininosuccinate lyase	4.3.2.1	Glycerol kinase	2.7.1.30	Pyruvate kinase	2.7.1.40
Arginase	3.5.3.1	Glycerolphosphate dehydrogenase (mitochondrial)	1.1.99.5	Succinate dehydrogenase	1.3.99.1
Histidine pyruvate aminotransferase	None	Guanine deaminase	3.5.4.3	*Alcohol dehydrogenase*	1.1.1.1
Phenylalanine pyruvate aminotransferase	None	Fructokinase	2.7.1.4	Xanthine oxidase	1.2.3.2
Glycerol-3-phosphate dehydrogenase	1.1.1.8	Glycollate oxidase	1.1.3.1	Glutathione peroxidase	1.11.1.9
NADPH-cytochrome *c* dehydrogenase	1.6.99.2	Cytochrome oxidase	1.9.3.1	*Catalase*	1.11.1.6
Isocitrate dehydrogenase (NADP)	1.1.1.42	UDP glucuronyltransferase	2.4.1.17		
Alcohol dehydrogenase	1.1.1.1	Microsomal, *N*-demethylase	None		
Riboflavin kinase	2.7.1.26	Pentobarbital hydroxylase			
FAD pyrophosphorylase	2.7.7.2				
Catalase	1.11.1.6				

The times of appearance of the three enzyme groups in the development of the rat, for example, are a late foetal period, a neonatal period and a late suckling period. It can be argued (Greengard, 1971) that the late foetal group represent an enzymatic preparation for the biochemical changes involved in birth, while the neonatal group are those that appear in direct response to birth and are not critically required immediately on parturition. Obviously, both the supply of nutrients and detoxification and removal of waste products is accomplished via the placenta and maternal circulation prior to birth, but suddenly at birth the newborn mammal must cope with both of these problems in new and independent ways.

Of course it might be argued that these observations on changes in the activities of liver enzymes are a function of the altering cellular composition of the liver. In particular, the liver is an important erythropoietic organ in the foetus and, on the 17th day of gestation, the number of pre-erythroid cells in the liver of the rat may exceed that of the hepatocytes. Actually, on a mass basis, the liver at that time is still mainly composed of hepatocyte cells. But the strongest argument that the changes in enzyme levels are not simply a function of changes in liver cell population, is that the dramatic increases observed are not reflections of obvious synchronous changes in cellular composition.

On the positive side of the argument is the observation that many liver enzymes appear in large amounts just about the time that their activity becomes necessary. Such an example is provided by the enzymes of the urea cycle, these proteins clearly becoming essential to the new excretory processes of the newborn in the conversion of ammonium ions (derived from amino acids by deamination) into urea. It is, therefore, impressive to note that amongst the late foetal enzymes are the urea cycle enzymes, arginase, arginosuccinate lyase, arginosuccinate synthetase, ornithine carbamyltransferase and carbomylphosphate synthetase I. Similarly, the neonatal cluster includes phosphoenolpyruvate carboxylase, which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, an important aspect of the citric acid cycle and the utilization of glycogen in glucose production.

Of course, many of these liver proteins are actually produced in response to signals received from outside the liver, particularly to hormones and other circulating informational molecules. But it should be stressed that many of these enzymes are not confined to the liver, and that although some appear synchronously in other tissues, others do not. For example, as pointed out by Greengard (1971), glucose 6-phosphatase appears in both liver and kidney in the neonatal cluster but glycerol 3-phosphate dehydrogenase develops prenatally in the liver and postnatally in brain and cytochrome oxidase appears chiefly in brain at the late suckling stage but neonatally in liver. Table 3 also reveals that some enzymes appear in two separate situations, such as catalase in the late foetal and the late suckling cluster. Actually, this enzyme persists at high levels into adult life but is present in very low concentrations just neonatally. Moreover, some enzymes occur in relatively high concentration in foetal life but decrease to very low levels during late foetal and adult life.

1. Hormonal control of liver enzymes

Studies of liver enzymes during development provide some interesting examples of hormonally modulated genetic expression. A variety of different hormones, both polypeptides and steroids, affect liver synthesis, the most important being glucagon, hydrocortisone and thyroxin. As discussed by Trueman (1974) most of the enzymes are regulated by one hormone only, but some, such as glucose-6-phosphate and arginase, are affected by two, glucagon and thyroxin, and hydrocortisone and thyroxin respectively.

One liver enzyme which responds dramatically to hormone is tyrosine aminotransferase (TAT), being stimulated by the pancreatic hormone glucagon. Curiously enough, the response of the liver cells in terms of TAT synthesis is not apparent until day 20 in the development of the rat. Foetal liver cells of the rat, if exposed to glucagon on day 17, produce no TAT in response. The particular lesion in the young foetus appears to be not the specific inability to synthesize TAT, but a lack of cyclic AMP, the presence of which is essential to the cellular response to glucagon. Since glucagon is a small polypeptide hormone, secreted by the pancreas (see Section 4A), a cellular response to its presence involves cyclic AMP. If cyclic AMP is administered to the foetal rat, TAT synthesis can occur earlier than the 20th day of gestation (Greengard, 1971). Actually, the amounts of circulating glucagon in the foetal rat are low until about 22 days of gestation, chiefly because the response of glucagon production to hypoglycaemia does not appear until that time. It is, therefore, clear that TAT production in the developing foetus is gradually scaled up as more factors in the regulatory process become active and after birth TAT synthesis comes to be influenced by cortisone and other hormones in addition to glucagon. The complicated control of TAT synthesis which characterizes adult metabolism is, therefore, pieced together slowly during development.

2. Inducible enzyme synthesis in liver

No eukaryotic systems display the impressive aspects of enzyme induction found in prokaryotes, where a preinduced state of less than one molecule/cell may be rapidly converted to large scale production of the appropriate protein. Indeed, only a few eukaryotic proteins are inducible in specific fashion, that is without an accompanying increased production of many other proteins. A list of relatively confined synthetic responses to inducers is provided by Tomkins and Martin (1970).

A close study of the mechanism of induction of one liver protein has been made by Tomkins and his colleagues (Tomkins *et al.*, 1969) in their work on TAT synthesis in cultured hepatoma cells. These cells are malignant liver cells isolated from the rat and can be grown freely in tissue culture. Although small amounts of TAT are synthesized by these cells normally, addition of a synthetic steroid, dexamethasone phosphate, leads to a dramatic increase in TAT activity and immunological assays reveal that the increased TAT enzyme activity is truly a reflection of increased enzyme synthesis. The effect is specific, moreover, since no accompanying increase in synthesis of other cell proteins is detected. When these cultured cells were synchronized with respect to the cell cycle, Tomkins was able to show that induction by the steroid occurred only just before or just after mitosis and not at any time in the cell cycle. In addition, removal of the inducer led to a cessation of enzyme synthesis.

Perhaps the most interesting aspect of Tomkins' experiments involves his use of the antibiotic Actinomycin D in this system. When Actinomycin D is applied before or during the induction phase, it effectively prevents induction, which is not surprising. But what is certainly remarkable is that provision of Actinomycin D after induction is effective in maintaining TAT synthesis even after withdrawal of the inducer, implying that RNA synthesis is not only necessary to accomplish induction but also to abolish it. Indeed, Tomkins *et al.* (1969) have shown that provision of Actinomycin D, even after the reduction of TAT synthesis following removal of inducer, can actually bring about an increase in TAT activity. Many schemes can be invented to account for these interesting phenomena, the simplest of which involve post transcriptional regulation of TAT by RNA intermediates.

The suggestion of Tomkins (Tomkins *et al.*, 1969) is that a separate regulator gene existed for the TAT enzyme, the product of the regulator gene being a repressor which either inhibited translation of TAT mRNA, or stimulated its breakdown, or both. The hormonal induction would then be seen as a combination with and inactivation of repressor. The role of Actinomycin D in preventing de-induction would then be viewed as interfering in the synthesis of the repressor substance by the TAT regulator gene. Prior to induction, the constitutive production of small amounts of TAT would be seen as a hormonal activity of the structural TAT gene and constant but not complete repression of TAT mRNA translation by the regulatory gene product. Tomkins *et al.* (1972) have discussed their results in the light of many other similar systems and this reference provides an interesting list of examples where inhibitors of macromolecular synthesis, including Actinomycin D, actually stimulate specific protein synthesis. They argue that simple interference with the enzymatic degradation of TAT, and other proteins cited, is an unlikely explanation and they have demonstrated experimentally that the rate of TAT degradation is not specifically affected by Actinomycin D.

Tomkins model, restated briefly, is that TAT production involves both a structural and a regulatory gene, both of which function only during a limited part of the cell cycle. The product of the regulatory gene is a labile molecule with the property of inhibiting the translation of the TAT messenger RNA produced by the structural gene. The regulator gene product, the repressor, may also promote TAT mRNA degradation. Although the role of the steroid hormone is less certain, it is presumed to interfere with the action of the repressor molecule, either by inactivating it through binding, or by a direct effect of the hormone, or the hormone/repressor complex, on the regulatory gene.

3. Enzyme synthesis in amphibian liver

The enzymes of amphibian liver have also attracted the attentions of those interested in the regulation of protein synthesis and they have been discussed by Trueman (1974) and more extensively by

Cohen (1970). The hormonal regulation of amphibian muscle proteins is also discussed by Tata (1971).

One interesting example of induced enzyme synthesis in the amphibian liver is the urea cycle enzyme carbonylphosphate synthetase I (CP-synthetase), which is chiefly a mitochondrial enzyme. The enzyme is inducible by thyroxine, a hormone which plays a crucial role in amphibian metamorphosis. Premature treatment of bullfrog tadpoles with thyroxine leads to premature appearance of this enzyme (Cohen, 1970). Thyroxin has many diverse effects in the induction of amphibian metamorphosis and it is certainly far from clear that it acts as a primary inducer of CP-synthetase. However, amphibian liver does provide an interesting alternative to mammalian liver as a system in which to study coordinate enzyme synthesis and the mechanisms of hormonal induction.

4. *α* foetoprotein in liver

α foetoprotein (*α*FP) is a major protein of the serum of many mammals in embryonic and early postnatal life. It is present in very low concentration in the normal adult, but in greatly elevated quantities in the serum of individuals suffering from hepatocarcinomas and teratocarcinomas.

The site of *α*FP synthesis in the embryo is the yolk sac and the foetal liver and probably the liver tumour cells in the affected adult. The site of the low level synthesis in the normal adult is presumed to be liver. This modulation of expression of the *α*FP gene or genes in the liver has been studied by a number of workers, particularly in terms of detecting levels of *α*FP messenger RNA. Koga *et al.* (1974) have extracted polysome RNA—which is chiefly mRNA, presumably—from the livers of foetal and adult mice and then assayed the mRNA population in this RNA by determining the *α*FP synthesis when the mRNA was mixed with a ribosomal cell free extract from either the foetal or adult mouse liver. Foetal liver mRNA with foetal liver extract gave a ratio of *α*FP to albumin of two or three to one. When adult liver extract was employed, a similar ratio of synthesis was obtained. If an adult liver mRNA preparation was employed, however, both foetal and adult liver extracts gave ratios of 0·2 to 0·6, with the amount of albumin synthesis relatively unchanged.

These experiments point to a great reduction in *α*FP mRNA production in the adult, as compared to the foetal, liver cell, although selective post transcriptional control by specific *α*FP mRNA breakdown cannot be ruled out. *α*foetoprotein, therefore, provides an interesting example of probable transcriptional rate control modulation which deserves scutiny at the level of the gene. It also seems important to establish whether the production in the normal adult is high production by only a few cells or low production by many or most liver cells. Even in the foetal liver, the distribution of the *α*FP amongst the hepatocyte population is not clear.

5. Comment

Liver is a very complex organ biochemically and its cellular composition is far from homogeneous. However, its very complexity does mean that the effects of ontogenic changes can be compared for different liver proteins. In addition, its susceptibility to hormonal influence provides an excellent example of eukaryotic gene expression mediated by hormonal effect.

The role of glucagon in TAT synthesis will surely continue to be the best model for the study of transcriptional and post transcriptional regulation by hormones, at least in mammals.

H. DNA-associated proteins

Our interest in DNA-associated proteins is twofold. First, because, as gene-coded products, they reveal some interesting aspects of gene expression and second, since their association with the DNA in chromatin is itself implicated in the control of the genes. This book is chiefly about eukaryotic cells and their genetic systems, and bacteria do not possess chromosomes in the conventional sense—the bacterial genome of DNA is not conjugated to basic protein but is sometimes referred to as a chromosome. It does, of course, have a relationship with acidic proteins, including both polymerase enzymes and repressor substances. We will, therefore, discuss the proteins associated with prokaryotic DNA in this section alongside the more lengthy comment on the proteins associated with eukaryotic DNA. It should also be pointed out that the eukaryotic chromosome is, in most cells and organisms, a temporary affair, at least visually, but there is no reason to believe that the proteins associated with DNA in chromosomes differ radically from those associated with the DNA in interphase chromatin. The title of this chapter, therefore, refers to the DNA associated proteins of both mitotic chromosomes and interphase chromatin.

It is conventional and convenient to divide DNA-associated proteins into two classes on the basis of their net electric charge—basic or acidic. The basic proteins of most eukaryotic cells are histones, but that found in sperm is protamine, or some other basic protein. Most cellular proteins are, of course, acidic and the acidic proteins of the cell are extremely diverse. Acidic proteins associated with the DNA are no doubt somewhat less diverse than the general cellular population, but they are still likely to be very variable in form and function. They include the polymerase enzymes associated with DNA during replication and transcription (and probably at other times too), as well as acidic proteins which are thought to be regulatory, as are, for example, the repressor proteins of prokaryotic operons. They also include other acidic proteins with enzymic or structural functions not encompassed by these two categories.

1. Histones and other basic proteins

Histones are associated with DNA in almost all eukaryotic cells. The sperm of many invertebrates and fish contains protamine, a basic protein very rich in arginine (about 68% of all protamine amino acids). Some sperm, e.g. that of carp, sea urchin and most plants, contains histone similar or identical to that in the somatic tissues, while the sperm of many insects and mammals contain yet another basic protein unlike histone or protamine. The reason for histone replacement in sperm is thought to be that the smaller size of the protamine molecule and its enrichment with arginine permits a tighter packaging of the DNA/protein complex within the sperm head (Feughelman *et al.*, 1955).

Since pure DNA carries a high net negative charge, due to the concentration of phosphate groups along the chains, and because one negative charge repels another, tight packing of pure DNA is unlikely. DNA is often packaged, at least loosely in bacterial cells and tightly in the heads of phages and within DNA viruses, implying that some electrical neutralization must occur in these situations. According to Ames and Dubin (1960), this is accomplished partly by positively charged magnesium ions and partly by polyamines such as spermidine and spermine. Other proteins, along with the small polyamine molecules, may be involved in DNA stabilization within the phage head (Kaiser *et al.*, 1963).

There are reports of an absence of basic protein in the chromatin of some dinoflagellates (Ris, 1962), cellular slime moulds and some fungi including *Neurospora* (Leighton *et al.*, 1971). However, the small size of the genome in these cells makes chromatin extraction particularly difficult and the absence of basic protein from these cells is, perhaps, best regarded as uncertain. Early reports of an absence of basic protein in yeast chromatin have now been contradicted (Wintersberger *et al.*, 1973) and some doubt cast on the lack of basic protein in *Neurospora* (Hsiang and Cole, 1973). Some of the fungal histones which have been isolated appear to be considerably different from the normal histones of animal and plant cells.

2. Histone structure and function

Table 4 indicates the characteristics of the main classes of histone isolated from normal cell—in this case calf thymus tissue. There are five main classes, signified differently in the terminologies of the different authors and each of these classes accounts for roughly 20% of the total histone in the cell (see Fig. 12). There is a tendency for particular ends of individual histone molecules to be either basic or acidic, although F1 histone has both ends polar and the middle piece hydrophobic, and it seems likely that the more basic parts of the molecule are those which are associated with the DNA (Comings, 1972). The question about how homogeneous particular histone fractions are is complicated by their methylation, acetylation and phosphorylation. Much or all of this aspect of histone structure is at least partly independent of primary structure or is superimposed on it and may result from post translational modification within the cell (Jergil *et al.*, 1970). It has been frequently suggested that such modifications of histone structure may be implicated in the binding of histone to DNA (Allfrey, 1969).

One histone, termed histone V or F2C in differing terminologies, has been isolated only from the nucleated erythrocytes of birds and amphibians (Neelin, 1964) and may have a special role in the extreme condensation of the chromatin in these nuclei (Brasch *et al.*, 1974). Histone I and V are very similar to one another and an evolutionary relationship is suggested (Greenaway and Murray, 1971).

The idea that histones themselves are the specific regulator molecules which control gene activity now has few supporters,

TABLE 4. Characteristics and classification of histones (from Comings, 1972).*

Terminology	Lysine-rich		Moderately lysine-rich		Arginine-rich	
Rasmussen *et al.*	Ia	Ib	IIb1	IIb2	III	IV
Johns and Butler	f1	f1	f2a2	f2b	f3	f2a1
Iwai *et al.*	Alanine-rich, very lysine rich		Leucine-rich inter-mediate	Serine-rich Slightly lysine-rich	Glutamic-rich Arginine-rich	Glycine-Arginine-rich (GAR)
Panyim and Chalkley	1	1	4	3	2	5
Biochemistry						
Lysine–arginine ratio	22		2·5	2·5	0·8	0·7
Percent total histone	20		18	24	20	18
Molecular weight	21,000		13–15,000	13,774	13–15,000	11,282
Number of residues	ⓐ212		136–145	125	130–145	102
N-terminal	Acetyl-serine		Acetyl-serine	Proline	Alanine	Acetyl-serine
C-terminal	Lysine		Lysine	Lysine	Alanine	Glycine
Probable DNA binding site	C-Terminal portion			N-Terminal portion	C-Terminal	N-Terminal portion
Sulfhydryl groups	None		None	None	Present	None
Acetyl groups	None		±	None	Two	Several
Methylated groups	−		−	None	One	20 lys in cth
Substrate for protein kinase phosphorylation	+++		±	+++	+	+

* Predominately calf thymus histone.

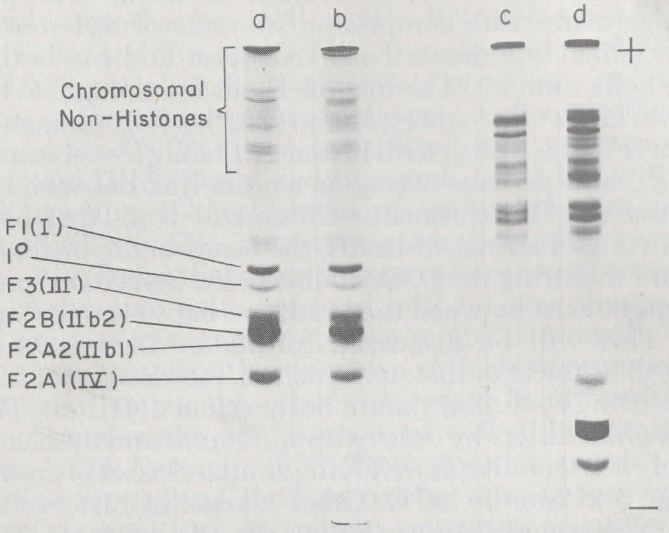


FIG. 12. Polyacrylamide gel electrophoresis of chromatin proteins from cerebellar and pituitary tissue. On the left are two gels in which the electrophoresis in urea buffer has separated both histone and non-histone proteins from (a) cerebellum and (b) pituitary. Note the similarity of the histone bands. On the right are two gels, both of cerebellar chromatin protein, but the one (c) an SDS extract of acid extracted chromatin and (d) salt extracted chromatin run in 7 m urea. Both of these gels demonstrate the heterogeneity of the non-histone proteins (from plate kindly provided by Professor Huang and reproduced with permission of the Society for Experimental Biology).

although there can be no doubt that histone or other basic protein is often involved in a rather generalized condensation of chromatin which may itself restrict or prohibit transcription. Reduction of transcriptional activity by conjugating DNA with histone *in vitro* is well established (Smart and Bonner, 1971), but the latter authors and others have now shown that it is rather difficult to demonstrate that one class of histones is more important than another in general chromatin repression. Much seems to depend on the ionic conditions.

If histones are not specific gene regulator molecules—their lack of heterogeneity certainly argues strongly against such a role—what is their role and how are they arranged within the chromatin substructure? Recent evidence (Kornberg and

Thomas, 1974; Kornberg, 1974; Baldwin *et al.*, 1975) points to a conjugate structure comprising subunits of different histone classes, which acquires a bead like form and binds the DNA double helix round it. The histone beads are thought to be made up of two kinds of tetramers, one an $(F2A1)_2(F3)_2$ tetramer and the other an $(F2A2)_2(F2B)_2$, with histone F1 being loosely attached to all beads, and perhaps acting as a cross link between beads in condensation of the chromatin. Chromatin would then be viewed as consisting of strings of beads, the beads being histone conjugates and the string the DNA double helix. Some non complexed DNA might exist between the beads, perhaps even the structural genes. This will be discussed further in Section 7A. Before leaving the subject of histone structure, their remarkable conservatism during evolution should be mentioned. Histone IV in calf and pea plant differs by only two amino acids and the changes are relatively conservative as involving similar classes of amino acids (Delange and Smith, 1971). Other classes of histones are also shown to be remarkably similar in calf and pea plant by Delange (1971) and other workers elsewhere. The extreme conservatism of the histones must imply some important principles governing their structure and function. The first is likely to be that their entire amino acid sequence is important for their role, permitting little or no evolutionary deviation. The second is that their role cannot easily be interpreted as binding to DNA on the basis of its base sequence, since considerable changes occur in the composition and arrangement of DNA bases, even between different species. On the face of it, the easiest explanation which squares with present knowledge is that histones are conservative partly because of crucial binding of one histone to another in the formation of mixed-histone conjugates and partly because of their binding to the DNA itself. It is not easy to see at the present time why histone should require such exact conformity to a standard amino acid sequence, however, if the role of histone in chromatin is largely a structural and supporting one. If it is implicated in the attachment of acid proteins, either regulator molecules or polymerase enzymes, then perhaps such a function would demand conservation of primary structure.

3. Histone genes

The genes coding for histone are noteworthy, chiefly because there is strong evidence that they exist as multiple copies.

Isolation of presumptive 9S histone messenger RNA from sea urchin polysomes has been followed by RNA/DNA hybridization studies (Kedes and Birnstiel, 1971). Annealling of this RNA to sea urchin DNA revealed relatively low *Cot* values, indicating a repetition of the histone genes to the extent of some 400 copies. The sea urchin 9S RNA also hybridized well to *Xenopus* and *Rhyncosciara* DNA (Kedes and Birnstiel, 1971), but with rather higher *Cot* value. If the histones of sea urchin, *Xenopus* and dipterous flies are near identical (as are those of pea and calf), these results suggest that some change in base composition of the histone genes may have occurred without any change in the histone amino acid sequence (Comings, 1972). Actually, there is evidence to suggest that the repetition of the histone genes is less in mammals than in the other eukaryotes mentioned but the number of gene copies per histone type will still be greater than one. Since the histone mRNA/DNA hybrids require a high temperature for melting, the base pairing must be very accurate, which again argues for homogeneity amongst the repeated copies of these genes. Such conservation of the base sequence of repeated copies also appears with the ribosomal RNA sequences and suggests that there may be a gene matching process involved (see also discussion in Section 3A on gene matching relevant to the immunoglobulin genes).

Histones are perhaps exceptional proteins in one other regard, namely that, unlike most other types of messenger RNA so far isolated from eukaryotes, histone messenger RNA has no tail of polyadenylate residues (Adesnik and Darnell, 1972). Some viral RNA species are also reported as lacking poly-A sequences (Stolzfus and Banerjee, 1972). As discussed in Section 3C in the context of globin synthesis, globin messenger RNA with and without poly A sequences is translated with equal efficiency but the life of the molecule may be reduced without the poly A attachment (Huey *et al.*, 1974). This has led to the suggestion that the poly A sequences are used as a ticketing system, determining the half life of the molecule (see discussion in review by Mathews, 1973). Perhaps histone mRNA survival is operated in some other way. The half life of histone mRNA is known to be exceptionally short (Craig *et al.*, 1971).

The production of histone mRNA, with an S value of about 7-9S, has proved to be of great value (Adesnick and Darnell, 1972; Kedes and Birnstiel, 1971; Borun *et al.*, 1967). Its isolation is largely due to an interesting and unusual fact about histone synthesis, namely that it occurs only during the S phase of the cell cycle (Robbins and Borun, 1967), while the synthesis of all other proteins is more or less continuous throughout the cell cycle. The polysomes synthesizing histones are also very small, often comprising three to seven ribosomes only (Moar and Nemer, 1971) and this observation has also facilitated the isolation of histone mRNA from polysomes. Relatively convincing translation of presumptive histone mRNA has been achieved by Jacobs-Lorena *et al.* (1972) and Breindl and Gallwitz (1973). Unlike globin mRNA, histone mRNA does not seem to be represented in the Hn RNA fraction.

4. Structure of histone in chromatin

A useful discussion of this topic will be found in Lewin (1974b). A model for the coiling of DNA with histone has recently been proposed by Bartley and Chalkley (1972, 1973). Most of the thinking on this topic has visualized the DNA arranged as a coiled coil, the histones taking up a partially helical form and being bound at least partly in the grooves of the DNA helix. If, as is now suggested by recent findings, different histones conjugate together to form large beads, then many of the earlier models become redundant. Since the acidic proteins are also involved in chromatin structure, the overall complex of chromatin will be discussed later, following our examination of acidic proteins in this chapter.

5. Non-basic proteins

As we have already emphasized, the non-basic proteins associated with DNA are of different kinds which can be broadly categorized as RNA polymerase enzymes, DNA polymerase enzymes and other proteins including regulatory proteins.

6. DNA polymerase

DNA polymerase enzymes remain a poorly understood topic, particularly in eukaryotic cells, and their association with chromatin may be relatively brief. In prokaryotes, three separate proteins have been isolated which display properties of DNA

polymerization. The first is DNA polymerase I, often termed the Kornberg enzyme since it was originally isolated and studied by Kornberg (see reviews, Kornberg, 1969, 1974). This enzyme is extremely active in bacterial lysates and tends to obscure other DNA polymerases present. It almost certainly normally functions as a repair enzyme, although the possibility of some genuine DNA synthesis by this enzyme has not been entirely excluded. A discussion of all prokaryotic DNA polymerases will be found in Lewin (1974a). A second enzyme, DNA polymerase II has been isolated from bacterial cells and found to resist antisera raised against the Kornberg enzyme. It is an active component of the baterial membrane system and since these certainly appear to be likely sites of DNA replication, it is possible that the main DNA replicase of the cell is localized on these membranes. At the moment, the function of DNA polymerase II *in vivo* is uncertain and it may be another repair enzyme. DNA polymerase III of prokaryotes is a relatively scarce molecule. An *E. coli* cell contains approximately 400 molecules of polymerase I, 100 molecules of polymerase II and only about 10 molecules of polymerase III (Lewin, 1974a). However, it is a very fast enzyme, adding some 15 000 nucleotides/min at 30°C, which probably renders the ten molecules adequate for the main replication of the cellular genome. Further work is necessary to confirm the suspicion that the third enzyme is the main DNA replicase of the bacterial cell.

Eukaryotic DNA polymerases are less well characterized than those of prokaryotes. Two separate DNA polymerases have been separated from extracts of mammalian cells, one chiefly nuclear and the other present in both nucleus and cytoplasm. Which, if either, is the main cellular replicase is not known. There is some evidence for a movement of DNA polymerase enzyme from cytoplasm to nucleus just prior to DNA replication (Littlefield *et al.*, 1963). It is, therefore, probable that the enzyme is synthesized in the cytoplasm, moves into the nucleus prior to S and is either degraded following its use in S or is chiefly exported to the cytoplasm after that time. There is some evidence from developing sea urchin eggs that DNA polymerase may remain bound to chromatin following the S phase (Fansler and Loeb, 1972), but most attempts to isolate DNA polymerase enzyme from eukaryotic chromatin or chromosomes have given very low yields or complete lack of enzymatic activity. The enzyme can hardly be regarded as an important regular constituent of chromatin.

7. RNA polymerase

RNA polymerase enzyme is a more permanent associate of the DNA than is DNA polymerase. In prokaryotes, it is a large multisubunit molecule with a total molecular weight of about 500 000. Since the molecule is required to have specificity for promoter, initiator, nucleoside triphosphate, nascent mRNA chain and terminator sites, its complexity is not surprising. Bacterial RNA polymerase is much better characterized than any of the eukaryotic RNA polymerases, especially that of *E. coli* (Burgess *et al.*, 1969). It consists of five subunits α, β, β', σ and ω (molecular weights 41 000, 155 000, 165 000, 86 000 and 9000 respectively) in the combination $\alpha_2, \beta, \beta', \omega_2, \sigma$. Of these subunits, the β subunit is involved in binding to DNA, the β' subunit in an interaction with rifampificin (an antibiotic substance discussed below) which suggests a correlation with initiation and the σ factor is involved in stabilizing the promoter/polymerase association at initiation, while the function of the α and ω units are presently uncertain. Discovery of the σ factor led to the suggestion that transcription could be controlled by a variety of such factors with different promoter region affinities but, except in the case of certain phages (Stevens, 1970) in which phage specific ω and σ subunits replace the bacterial ones in the structure of the RNA polymerase, no such heterogeneity has been detected (Bautz and Bautz, 1970). Actually, there is now good evidence that σ factor operates not so much by increasing the affinity of the polymerase for one particular promoter sequence, but by decreasing its affinity for the non-promoter sequence in DNA (Mueller, 1971).

No example of transcriptional changes being mediated by polymerase modification are known in eukaryotic cells, although bacterial polymerase sigma factor is reported to stimulate eukaryotic RNA polymerase when injected into *Xenopus* oocytes (Tochini-Valentini and Crippa, 1970). A protein of molecular weight about 70 000 has been isolated from rat liver and calf thymus tissues (Seifart, 1970) which appears to specifically stimulate one of the eukaryotic RNA polymerases on double stranded DNA templates. It is suggested that the association with the enzyme may alter its conformation and hence its affinities, but more evidence is needed before it can be equated with the sigma factor of prokaryotic RNA polymerase.

Eukaryotic RNA polymerases are of three kinds, termed peak I, peak II and peak III enzymes, of which the first two predominate (Roeder and Rutter, 1970). Peak I enzyme is now termed RNA polymerase A and peak II enzyme RNA polymerase B. They have different locations within the cell, the type A enzyme being chiefly associated with the nucleolus and type B with the extra nucleolar chromatin. The fact that these locations reflect their specialization is borne out by their susceptibilities to certain antibiotics. The type A enzyme is thought to be chiefly involved in synthesis of ribosomal RNA and this is supported by its almost total inhibition by Actinomycin D at low concentration, i.e. 0·5 μ gm/ml (Moore and Ringertz, 1973). On the other hand, the type B enzyme is specifically inhibited by α amanitin (Lindell *et al.*, 1970). Interestingly, this compound also inhibits bacterial RNA polymerase, but rifampicin, another antibiotic compound which inhibits the bacterial enzyme, shows little or no effect on eukaryotic RNA polymerases. A prediction might be that mitochondrial RNA polymerases, when isolated and studied, would show many similarities to the prokaryotic enzyme. But, actually, one eukaryotic mitochondrial RNA polymerase, that of the fungus *Neurospora*, has been studied (Kuntzel and Schafer, 1971) and turns out to be a single unit molecule of molecular weight of about 64 000. A similar enzyme has also been identified in bacterial cells infected with phase T7. The type III eukaryotic RNA polymerase referred to above is present in lower concentrations than the other two and is slightly, but not strongly, associated with nucleolar chromatin. There is some evidence that it may be mainly concerned with production of small RNA, probably transfer or 5S ribosomal.

The subunit structure of eukaryotic RNA polymerases remains unclear. Both A and B enzymes possess at least two subunits each (Kedinger *et al.*, 1971), but as many as five subunits have been found in the type B enzyme extracted from Hela cells (Sugden and Keller, 1973). The overall molecular weight of the eukaryotic enzymes seems to be the same as the bacterial enzyme, about 500 000 (Chesterton and Butterworth, 1971).

The amounts of RNA polymerase which are associated with chromatin are rather variable in different cells and tissues and are not always correlated with the RNA synthetic activity of the cell. For example, the nucleus of the *Xenopus* erythrocyte, which exhibits as little RNA synthesis as any eukaryotic nucleus (Maclean *et al.*, 1973) has a high content of RNA polymerase in its chromatin (Tata, personal communication). Despite this high content, the RNA synthesis of these nuclei, when isolated and incubated *in vitro*, is enhanced by the addition of exogenous bacterial RNA polymerase (Hilder and Maclean, 1974). It seems most probable, therefore, that RNA polymerases may be sequestered away in an inactive form or may act as structural proteins in chromatin, thus making addition of active enzyme necessary for increased transcription. A device for controlling RNA polymerase activity on chromatin could clearly be crucial in overall gene expression, especially in situations close to complete genetic shutdown.

8. Regulatory non-basic proteins

Many non-histone proteins besides polymerase enzymes are associated, at least in small amounts, with DNA in chromatin. They include DNAase, proteases, methylases, ATPase, lactate dehydrogenase, nucleotidyl transferase and others (Comings, 1972). Moreover, if non-histone proteins are isolated from chromatin and run on sodium dodecyl sulphate-polyacrylamide gels, some 10–30 bands can be resolved, with molecular weights ranging from 5000–100 000 daltons (Hilder *et al.*, 1975). There is also broad similarity between the band patterns from different organs and species (Elgin and Bonner, 1970). Unfortunately, the identity of the bands has not been established, although, presumably, many are accounted for by the proteins listed above. An observation that has been made in many laboratories is that, although histones do not apparently vary in type or amount between euchromatin and heterochromatin (however these fractions are defined), the amount of acidic protein is much greater in euchromatin than in heterochromatin. No doubt much of this increase may be due to RNA polymerase, but careful qualitative analysis of acidic protein, especially in the two types of chromatin, is obviously desirable.

The chromatin of calf thymus has the following composition, relative to the DNA content:

DNA	100
Histone	114
Non-histone protein	33
RNA	7

HeLa cell chromatin is richer in acid proteins, apparently (DNA 100, histone 115, non-histone protein 86 and RNA 12), and the amount of the non-histone protein may be much more than double this amount in mitotic chromosomes (Sadgopal and Bonner, 1970).

Some of the non-basic proteins of chromatin seem to be phosphorylated in the cell and this may provide some tissue specificity for certain of these proteins. Such a suggestion has been made by Kleinsmith and Allfrey (1969) and Teng *et al.* (1970). When non histone nuclear proteins are recovered from electrophoretic gels and tested for their binding ability to DNA, there is evidence for a relatively non-specific binding of some protein portions to DNA, while other bands show some indication of specific binding, especially between prokaryotic and eukaryotic DNA (Van den Brock *et al.*, 1973).

It is obvious then that the non-histone nuclear proteins are a complex of many different molecules, but that only a limited number of bands can be resolved from their electrophoresis (see Figs 13 and 14). Some of these bands are no doubt enzymes known to be present in chromatin. If non-histone proteins are involved in eukaryotic gene activation and repression at a relatively specific level, then we must assume that certain fractions which, because of their similarity of molecular weight, tend to appear in electrophoresis as one band, are really highly heterogeneous. It is also possible that their variability and specificity is increased by phosphorylation. Stimulus for the identification of nuclear acidic proteins having regulatory roles in eukaryotic chromatin has come from the discovery that repressor substances coded by the regulatory genes of bacteria are indeed proteins (see discussion in Chapter 2). Such proteins have now been isolated. The technique involved in their isolation was the utilization of an *E. coli* mutant which made large amounts of repressor and the transfer of its lactose operon to a defective phage so that, during phase replication in a bacterial cell, large amounts of repressor were released. Following induction of the operon with IPTG (isopropyl thiogalactoside), the protein was recovered bound to the IPTG inducer. Proof that the protein was indeed the repressor protein is given by its absence from cells known to have defective regulator genes, i.e. constitutive mutants. Such cells yield no protein which binds to the IPTG (Gilbert and Muller-Hill, 1970). The bacterial lac repressor is a tetrameric protein composed of four identical subunits, each of 38 000 daltons. Only about 10 lac repressor molecules are present in each bacterial cell. Repressor proteins have also been isolated for the tryptophan and arginine—operons in *E. coli.* Interestingly, although the tryptophan repressor is also present at about 10 molecules/cell, the arginine repressor is present in a concentration of about 200 molecules/*E. coli* cell (Urm *et al.*, 1973).

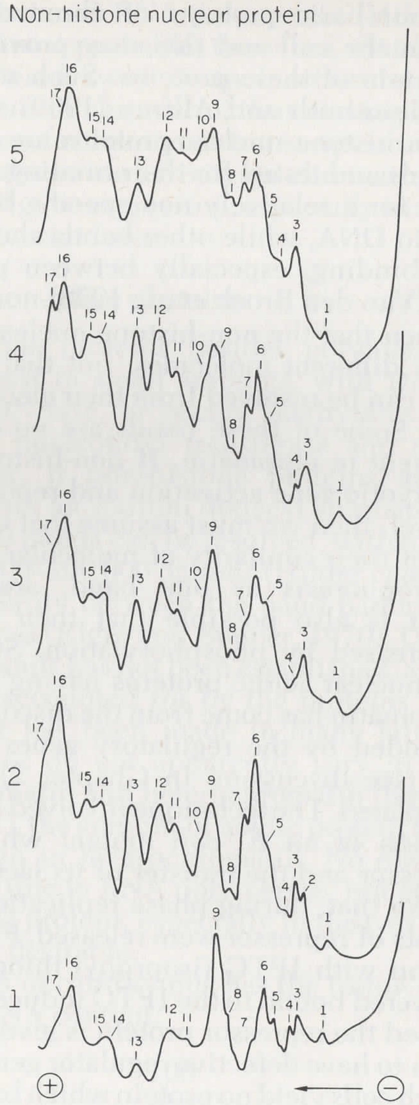
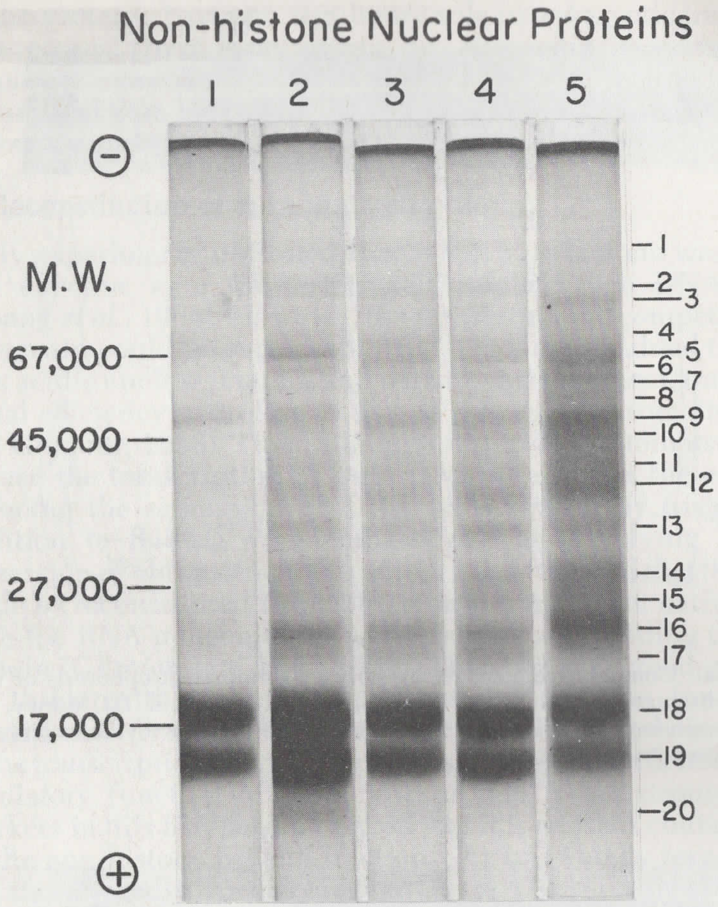


FIG. 13(a). Electrophoretic separation of non-histone nuclear proteins from duck erythroid cells at different stages of maturation. Prominent bands are numbered for comparison with the densitometric tracings in Fig. 13(b). Gel 1 is an extract of nuclei from mature erythrocytes, two from late polychromatic erythrocytes, three mid polychromatic erythrocytes, four early polychromatic erythrocytes and five from erythroblasts and some early polychromatic erythrocytes.



13(b). Densitometric tracings of non-histone protein bands seen in 13(a). The numbers refer to the prominent bands shown in Fig. 13(a). Photographs kindly provided by Dr. Ruiz-Carrillo (1974) and reproduced with permission of the American Society of Biology and Chemistry.

The search for specific gene regulators amongst the nuclear non-basic proteins of eukaryotic chromatin is hampered on the one hand by the small number of any one such molecule that might occur in a cell, and on the other by the relative lack of heterogeneity resolved by electrophoretograms of chromatin non-basic protein. For these reasons, the burden of evidence for

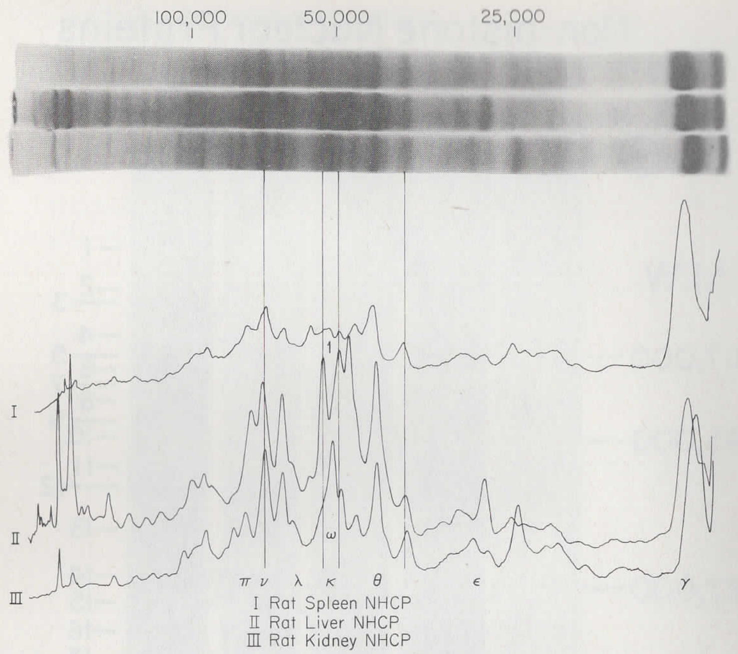


FIG. 14(a). Comparative analysis by polyacylamide electrophoresis of non-histone chromatin proteins from (1) rat spleen (II) liver and (III) kidney. The gels were run in an SDS-Tris-glycine buffer and are shown with the accompanying densitometric tracings.

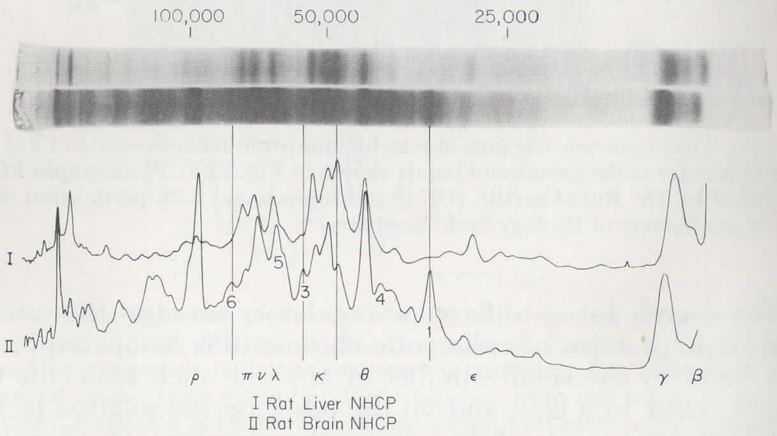


FIG. 14(b). As in 14(a) but comparing (I) rat liver with (II) brain. Note the extensive tissue specificity of the bands revealed (photographs kindly provided by Dr. Elgin).

acidic proteins having a regulatory role in eukaryotic transcription comes from experiments on chromatin reconstitution, involving extraction of DNA, histone and acid protein from tissues and then testing the fidelity of transcription of the DNA, following addition of the other fractions.

9. Reconstitution of eukaryotic chromatin

Early experiments indicated that DNA plus histone was much less effective as a transcriptional template than DNA alone (Huang *et al.*, 1964). However, the transcriptional competence of chromatin could be increased if the histone was added to DNA plus acidic nuclear proteins and with this arrangement, transcriptional efficiency was close to that of the native chromatin (Paul and Gilmour, 1968). Thus, although the acidic protein itself will reduce the transcription of pure DNA, it also has the effect of reducing the repression of DNA transcription by histone. In addition to finding a generalized role of modifying histone repression of chromatin, these authors also reported that the RNA made by reconstituted chromatin would compete for native DNA with the RNA molecules native to the tissue providing the acid protein (Gilmour and Paul, 1969). There are now good grounds for thinking that much of this competition was for highly repetitive DNA sequences, which are probably largely irrelevant to the transcription of structural genes, and so the evidence for the regulatory function of the acidic protein is not strong. Some workers in this field are convinced that it is an RNA contaminant of the non-histone protein fraction which is chiefly responsible for the allegedly specific regulation of transcription in these experiments (Bekhor *et al.*, 1969). It is, of course, almost impossible to entirely eliminate the possibility that the acidic protein fractions are contaminated by small amounts of RNA and so the possibility that chromatin associated RNA is the chief regulatory molecule cannot be entirely excluded. Recent experiments by Spelsberg *et al.* (1971) have demonstrated, very clearly, that in these reconstitution experiments the specificity of transcription is a property of the acidic protein fraction and not of the histone. Histones from different tissues seem to perform similarly in such experiments. Paul (1972) has used the evidence from chromosome reconstitution experiments to provide a general theory of eukaryotic chromatin structure.

By far the most impressive and convincing experiments involving specific regulation of eukaryotic structural genes are those of

Axel *et al.* (1973) and Barret *et al.* (1974). The first authors did not reconstitute chromatin, but compared the transcriptional activity of chromatin and DNA *in vitro*, in the presence of bacterial RNA polymerase. With the use of a DNA probe for globin RNA (made from globin mRNA with viral reverse transcriptase) globin RNA synthesis was detectable with reticulocyte chromatin, but virtually undetectable with liver chromatin or native DNA. The absence of reaction with the native DNA is explained by the authors in terms of restriction of transcription to globin genes in reticulocyte chromatin and to a generalized transcription with the DNA, implying very limited synthesis of globin sequences. This work provides very strong evidence for chromatin carrying the mechanisms for its own specificity, although it does not rule out the possibility that non-proteins are involved in the regulation. It also supercedes earlier experiments in proving transcription of structural genes, rather than repetitive DNA sequences. Perhaps the main reservation possible is one which the authors have laboured to exclude, namely that contaminating globin mRNA was present in the chromatin and accounts for the activity of the globin DNA probe. Barrett *et al.* (1974) have undertaken experiments with reconstituted chromatin, the reconstitution involving DNA and histone from chick reticulocyte and non-histone protein from either reticulocyte or liver. Globin mRNA transcripts were identified by hybridization with a radioactive DNA probe (made with reverse transcriptase on globin mRNA) and were detectable only when the non-histone protein was derived from reticulocytes, not from liver.

These experiments closely resemble the earlier work of Paul and his collaborators on reconstituted chromatin. As with the experiments of Axel *et al.* (1973), the major worry in interpretation is the possible contamination of the reticulocyte non-histone protein with globin mRNA sequences.

In my own laboratory, we have taken an alternative approach in attempting to derepress chromatin within the nucleus (Hilder and Maclean, 1974). Using nuclei from *Xenopus* erythrocytes, in which RNA synthesis is minimal, we are attempting to induce the synthesis of new RNA by exposing the nuclei to protein isolated from active chromatin or cytoplasm. Since there are grounds for believing that many systems of gene regulation in eukaryotes are operated by positive control (see Section 7A), the search for gene induction by regulatory proteins seems appropriate.

10. Comment

The proteins associated with DNA are a mixed bag and, to date, no authentic regulatory proteins have been identified in eukaryotic cells. Although histone now looks an unlikely candidate for a specific regulatory molecule, its association with DNA is important to the genetic expression of the DNA. An understanding of the architecture of DNA/histone is certain to narrow the field of possible eukaryotic mechanisms of gene regulation. The repetition of histone genes, conservation of histone sequences and peculiarities of histone mRNA make histone a most interesting model system in other respects also.

The search for regulatory factors amongst the nuclear acidic proteins has been scarcely more rewarding, although chromatin reactivation in artificial heterokaryons always provides tantalizing evidence that specific changes in transcriptional commitment are possible. Identification of globin mRNA synthesis in reconstituted reticulocyte chromatin will surely be a major step forward and the specific reactivation of erythrocyte nuclei *in vitro* may help to identify the regulatory inducers of eukaryotic structural genes.

5. Experimental Systems of Differential Gene Function in Eukaryotes—Systems Not Well Understood in Molecular Terms

A. The early mammalian embryo 184
B. Slime moulds 192
C. Protozoa—studies on cortical inheritance 200
D. *Acetabularia* 205
E. *Hydra* 215
F. The imaginal discs in *Drosophila* 221
G. Higher plants 233
H. Mitochondria and chloroplasts 241

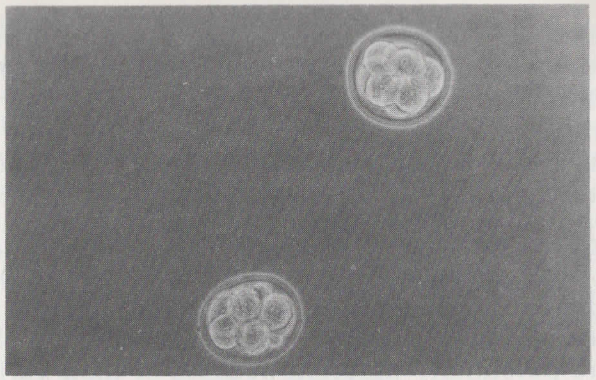
A. The early mammalian embryo

The events of cell determination and differentiation in the mammalian embryo, especially that of the mouse, have proved to be useful for studying gene expression. Of particular interest is the very early commitment of the embryonic cells to trophoblast or inner cell mass, probably the first act involving differential gene expression in mammalian development. The topic has been recently reviewed by Herbert and Graham (1974).

It should, first, be stressed that the mouse embryo can be cultured for periods up to seven days in Eagle's medium or jelly from the lens of the bovine eye. Moreover, embryos can be removed from the maternal uterus, even in the second half of pregnancy, and cultured in well-gassed circulating serum for up to two days without obvious change in the pattern of development (see New, 1971 and Fig. 15). These successful culture experiments seem to demonstrate that the role of the maternal uterus in determining satisfactory development is probably not a crucial one. It has also been found possible to obtain satisfactory development of mouse blastocysts by their artificial implantation into such tissues as the kidney and the liver and even into the male testis (see Kirby, 1965). So, as Herbert and Graham remark (Herbert and Graham, 1974), the mother is no more to the embryo than "a heart lung and kidney machine that is required to support, but not to control, cell differentiation in the developing embryo".

1. What determines cell fate in the mammalian embryo?

Assuming then that the factors which accomplish determination and differentiation in the mammalian embryo are all intra-



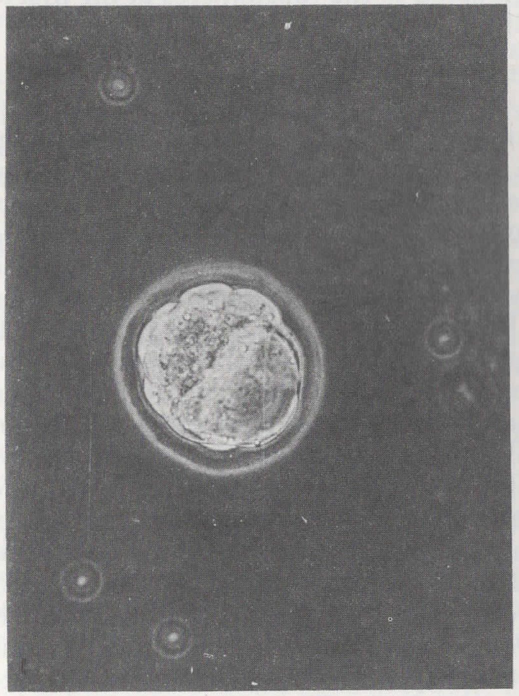


FIG. 15(a). Living 8-cell stage embryos removed from the oviduct of a mouse about 2½ days after mating.
15(b). Living mid-blastocyst stage mouse embryo showing the outer zona pellucida and a sphere of trophablast ectoderm with a group of inner-cell-mass cells partly filling the blastocoele (photographs kindly provided by Dr. A. E. Wild).

embryonic, it is possible to construct theories which could account for the differentiation and separate development of trophoblast and inner cell mass. Such theories have also been applied in modified forms to similar problems in non-mammalian embryonic development. Three main theories have been proposed and these can be stated in brief as follows;

1. Determination depends solely on cell position in the embryo, the inner cells becoming inner cell mass (ICM) and the outer cells becoming trophoblast.
2. The mammalian egg has reference points and the blastomeres are potentially different because of their variable relationship to these original reference points.
3. Morphogenetic factors occur in the mammalian egg and are unequally segregated to the blastomeres at the first and subsequent divisions.

Recent evidence on the applicability of these three theories to the mouse embryo has been reviewed by Herbert and Graham (1974) and the main weight of evidence favours the first theory. Of course, some place for the mechanisms envizaged by the other theories is not necessarily entirely excluded, but their importance does not seem to be paramount.

The body of evidence which suggests that cell position, inside or outside of the cell mass, chiefly determines cellular commitment, will now be briefly reviewed. First, up to about the 32 cell stage of development, at least some blastomeres remain totipotent and, if removed from the embryo or displaced within it, may alter their ultimate fate (Gardner *et al.*, 1973). The complementary observation is that, up to the eight cell stage, each blastomere is still totipotent and can go on to form at least both foetus and trophoblast. The distinction between inside and outside cells first becomes manifest at the 8–16 cell stages of mouse development, suggesting that it is this event which begins to limit totipotency. Additional evidence along these lines comes from the experiments of Mintz (1965) on the production of artificial mouse chimaeras. Mintz has been able to form whole blastocysts by fusing together pairs of 32 cell stage embryos. Cell marking of these embryos (Stern and Wilson, 1972) reveals that cells within these mixed embryos do not sort out to their original commitment to be inside or outside. Similar experiments have been carried out recently which have made use of marker genes such as coat colour and with the additional refinement that the cells from the two donor embryos were manipulated at fusion so that those from one embryo became outside and those from the other inside (Mintz, 1970; Mullen and Whitten, 1971). In these experiments, the results, although not unequivocal, strongly suggest that the cells placed on the inside of the chimaeric composite embryo are the ones which go to form the new foetus and that the outer cells become trophoblastic. There is additional experimental data which could be quoted, but the gist of all the current evidence is that cell position in the mouse embryo at about the 16 or 32 cell stage decides what the fate of the affected cells will be.

2. Are inner and outer cells different?

It might be assumed that because the cells of the mouse embryo are not determined by the 16, or even the 32 cell stage, the cells would all be morphologically identical. But this is not the case. The outer trophoblast cells differ on a number of criteria from those of the inner cell mass at this stage (see Fig. 15). In other words, there is apparent differentiation without true determination. Put in genetical terms, this means that some alteration of gene expression is detectable at this stage, but the cells retain great plasticity and are not actually determined in an irreversible way until a later stage. Let us examine the parameters on which the strictly undetermined cells of the 16 cell stage mouse embryo can be seen to differ, according to their location in trophoblast or ICM.

1. The inner cells divide faster, as judged by the rate of incorporation of tritiated thymidine into DNA (Barlow *et al.*, 1972) and assays of the actual cell number confirm that the differential labelling reflects differential rates of cell multiplication (Herbert and Graham, 1974).
2. When isolated and grown in culture the outer cells are found to form closed multicellular vesicles, with some evidence of cellular pumping leading to the collection of liquid within the vesicle. Cells from the inside of the blastocyst never show this property and remain as solid groups of cells in culture (Gardner, 1972).
3. Outside cells develop tight intercellular junctions in the early morula (8–16 cell stages), while, in contrast, tight junctions are not found between the inner cells, nor between the inner and outer cells.

4. There is some evidence that the pseudopregnant uterus responds differently by way of decidua formation to the two types of cells (Gardner, 1972).
5. Inner cells have a higher RNA content than the cells on the outside (Mulnard, 1971). There is also a suggestion that the two cell types differ with respect to the activity or localization of certain enzymes, e.g. acid and alkaline phosphatase, but the evidence is contentious and the observation not very penetrating. Any difference in rate of cell division, as discussed in (1), will bring other biochemical differences in its train.

All these differences have been listed and discussed by Herbert and Gardner (1974). Taken together they present the coherent idea that the cells inside and outside of the morula are different, i.e. that they are differentiated one from the other. Yet, it was established earlier in this chapter that these two types of cells are not actually determined, i.e. they do not vary, at this stage, in their developmental potential. Thus we have the interesting situation in which cells are differentiated but not determined.

Due to the conclusion that the inside and outside cells of the 16 day mouse embryo, though slightly different from one another, are not determined, it is appropriate to ask when determination does occur in these cells. Little information is available on this point. The considerable regulative ability of the inner cell-mass cells, even in the late blastocyst, is shown by normal development following the removal of between 5 and 15 000 μm^3 of cells from the inner cell mass. Conversely, trophoblast cells show many symptoms of determination by the 64 cell stage (Gardner *et al.*, 1973). At this stage, if cell groups are formed from trophoblast cells and introduced into the pseudopregnant uterus, little development occurs. In a few cases, some giant trophoblast cells have been recovered. Some trophoblast determination or fixation involves the polyploidization of trophoblast cells. It is doubtful if such cells can divide and so their potential as sources of further embryonic tissues would be nil. Trophoblast cells are, therefore, probably determined at or about the 64 cell stage, or at least their genetic potential is then severely restricted. On the other hand, the inner cell-mass cells remain highly regulative until much later.

3. Teratomas from embryonic cells

Teratomas are benign tumours consisting of a somewhat haphazard mixture of several adult tissues. They are probably always derived initially from embryonic cells, either from primordial germ cells in the testis or parthenogenically activated ova in the ovary. On the other hand, teratocarcinomas, or malignant teratomas, are highly malignant tumours which, in addition to the mixture of adult cell types, also retain some embryonal carcinoma cells that remain relatively embryonic in character and are probably the stem cells of the tumour. Since both teratomas and teratocarcinomas constitute situations in which rapid and varied differentiation from embryonic cells occurs, they are extremely interesting research systems. Their biology and experimental use have been reviewed by Pierce (1967) and Damjanov and Solter (1974).

In recent years certain fortuitous discoveries about teratomas have greatly increased their potential as experimental systems. Firstly, a mouse strain, strain 129, was discovered which had a high incidence of spontaneous testicular teratomas (Stevens and Little, 1954). Second, it was found possible to induce teratomas by inserting one-celled mouse eggs into the testis of a male mouse (Stevens, 1970). Third, it has proved possible to establish teratocarcinoma cells in culture. Some of these cell lines remain undifferentiated in culture but differentiate into numerous cell types following implantation into a new host (Kahan and Ephrussi, 1970). It has previously been demonstrated that a single embryonal cell would, on insertion into a new host, produce the whole spectrum of varied differentiated cell types already recognized in the teratocarcinoma (Kleinsmith and Pierce, 1964). Last, if mice are injected intraperitoneally with teratocarcinoma stem cells, the injected mice develop so-called embryoid bodies in the ascites fluid. These bodies have a morula-like appearance and will, if removed and grown on in culture, produce a variety of differentiated cell types—nerve cells, muscle cells, cartilage cells and so on (Damjanov and Solter, 1974).

Certain aspects of the growth and development of teratomas and teratocarcinomas are particularly informative in terms of gene expression. First, it should be noted that teratocarcinomas are tumours of embryonic tissue, as determined both by their origin and by their capacity for differentiation. This must surely make it plain that not all tumour cells are analogous to embryonic cells, nor can they be classed as undifferentiated. The stem cells of teratocarcinomas are, however, truly undifferentiated malignant cells. In other words, whatever special change in gene expression is involved in malignancy, it may occur in both relatively undifferentiated and relatively highly differentiated cell types.

The ability of the original embryonic cell of a teratoma and of the stem cell line of teratocarcinomas to differentiate into a variety of identifiable highly differentiated cell types is also intriguing. It implies that the genetic programmes of these cell types have all been differently selected within the very limited environment of the tumour site, which certainly provides a much more simplistic model of differentiation than many current views would envisage. How can the genetic programme for erythropoietic cells or neurones be selected for during the development of the teratoma? Actually, recent observations suggest that the step from embryonic stem cell to differentiated cell is not a direct one, but that a group of stem cells produce, by division, a small nest of cells which then collectively differentiates into one tissue cell type (Solter, 1975). This seems to imply that some modified and somewhat disorganized form of embryonic development occurs within the teratoma. In the teratocarcinoma, the added complexity would be that stem cells could continue to produce other undifferentiated stem cells by division, in addition to the nests of differentiating tissue cells. So, although differentiation is dramatic in these model systems, it is not totally unlike normal differentiation. But the potential usefulness of teratocarcinoma cells for studies on differentiation must be very great, particularly because they must surely, in time, provide a readily available study system for *in vitro* differentiation.

Only of passing interest here, but of great biological significance, is the observation that the immunological responsiveness of the host animal greatly influences the differentiation and malignancy of any introduced teratocarcinoma stem cells (Solter, 1975).

4. Comment

Early mammalian embryology has been selected for discussion in this book chiefly because of the evidence available on differentiation and determination of trophoblast and inner cell-mass cells in the early mammalian blastocyst. Such observations underline an important aspect of determination, namely that it may be preceded by at least partial differentiation. It is, therefore, important to ensure by suitable tests of developmental potency and regulation that a particular cell or tissue is truly determined, i.e. has a severe restriction placed on its potential genetic expression. Such a state cannot simply be deduced by its apparent differentiation. Although it is likely that, in most cases, determination precedes obvious cellular differentiation, it need not always do so. Some cells may be specialized and, therefore, restricted in their present genetic expression, without a concomitant restriction of potential genetic expression.

Experiments and observations on mammalian teratomas and teratocarcinomas are also revealing in terms of gene expression. These phenomena are examples of aberrant embryonic development, with differentiation of some cell types proceeding to the extremes found in the normal adults. They underline for us the fact that tumours of embryonic cells exist, which conversely implies that not all tumours are a return to an embryonic or undifferentiated cell type. Moreover, they imply that the particular genetic programme characteristic of the malignant state can be imposed on both embryonic undifferentiated and adult differentiated cells alike.

Teratoma studies hold great promise for the future. Satisfactory differentiation of teratocarcinoma cells in tissue or organ culture will surely become an increasingly useful experimental system and might well constitute the long sought for source and assay for differentiating factors *in vitro*. One such system has already been reported (Evans, 1975). It will be surprising if comparative studies on different teratocarcinoma cell lines do not reveal differences in developmental potential and particular conditions for particular types of tissue differentiation within them. Moreover, they seem to present a testing ground for the genetic loss theory of carcinogenesis. If the embryonic malignant cell is actually genetically deficient, this deficiency is not apparent in its production of apparently normal differentiated cells of a variety of types. Such derived cells are, in addition, not detectably malignant. This leads us to the suggestion that the normality of the differentiated tissues produced by teratocarcinoma stem cells deserves careful scrutiny, perhaps by their employment in tissue grafting experiments.

Attempts have already been made (Jami *et al.*, 1973) to fuse together teratocarincoma stem cells and fibroblasts and then assay for differentiation by injection into a new mouse host. Such experiments have, so far, yielded only fibrosarcoma-type tumours. Whether this can be explained by loss of certain genes from the hybrid cells or by a real repression of differentiated function cannot be deduced from the experiments. It is, however, a most useful and interesting experimental technique.

B. Slime moulds

Slime moulds are simple, though not necessarily primitive, eukaryotic cells. They comprise two different groups of organisms, which are almost certainly not closely related. The first group, the so-called cellular slime moulds, classified under the grouping of *Acrasieae,* includes one genus greatly favoured for research, *Dictyostelium.* The second main grouping is the *Myxomycetes,* of which the best known laboratory species is *Physarum,* the acellular or plasmodial slime moulds. These two unrelated groups, both unfortunately called slime moulds, have made special contributions to the subjects of cell biology, differentiation and cellular control of gene action. We will, therefore, discuss both here, but under separate sections.

1. The cellular slime moulds

These organisms have an interesting and fairly complicated life history. The single-celled motile vegetative cells are termed myxamoebae, although their relationship to the true amoebae is probably tenuous. They are to be found in woodland soil, feeding on soil bacteria in damp areas. Provided food remains available and humidity is high, this part of the life cycle will continue indefinitely. However, if food becomes scarce, the separate amoebae proceed to aggregate into a multicellular form (see Fig. 16), eventually adopting a discrete cylindrical shape termed a slug or grex. The manner and mechanism of the cell aggregation has interested biologists for many years; it has been known to be dependent on a chemical attractant for some time. The chemical attractant was originally termed acrasin, but the main component is now known to be cyclic AMP. Not all the amoebae secrete the cylic AMP, or at least not in the same amounts.

Once the aggregated myxamoebae have associated in sufficient numbers to form the slug, the cell mass then becomes capable of integrated movement and proceeds to wander about on the substrate and display a front and rear polarity. After a period of migration the slug ceases to wander and rounds up, the leading tip of the slug becoming a vertical tip. There now begins a series of morphogenetic cell movements destinated to produce a fruiting body. A stalk forms from the tip, the cells from the tip of the slug becoming stalk cells as they move in sequence up the partly formed stalk. Finally, as the stalk lengthens, the cells which have been at the rear of the slug ascend up through the stalk and proceed to differentiate into spore cells. Thus the cellular distribution within the slug is expressed in the final morphological formation of the fruiting body. On germination of the spores, an amoeboid cell emerges from each spore and, following a number of divisions, results in many new single celled myxamoebae.

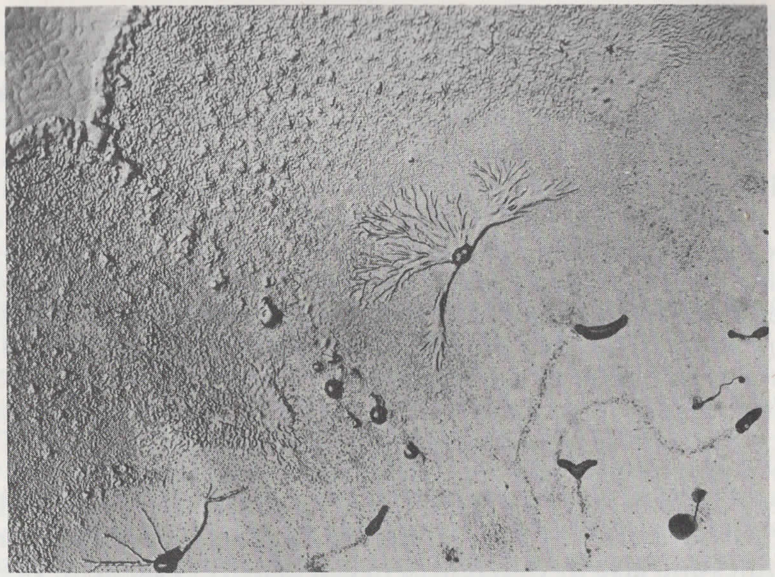


FIG. 16. Culture and differentiation of the slime mould *Dictyostelium discoideum.* The photograph shows a culture of these organisms on agar with part of a feeding front, in which the amoebae are actively feeding on bacteria, and aggregating grex with amoebae moving up the arms to the central cell mass, some complete mobile grexes, and some fixed grexes supporting fruiting bodies (photograph kindly provided by Dr. D. Garrod).

The form and life history of *Dictyostelium* has provided biologists with a most interesting model of cell differentiation and biochemical genetics, although the selection of mutants and actual gene mapping are still in a very preliminary stage.

One of the first aspects of the biology of *Dictyostelium,* which seemed open to experimental attack, was the mechanics of determination of cell type. Since the front of the slug provided the fruiting body stalk cells and the rear gave rise to the spore cells, it was reasonable to ask whether these cells were already committed while in the migrating slug. The answer is that they are, at least to some extent, already so committed (Gregg and Bodman, 1973). However, this preliminary commitment seems not to be unconditional at this stage. Dissection of a migrating slug into isolated subsections of front and rear extremities, commonly results in regulation of these cell clumps to form new slugs capable of fruiting body formation (although some fruiting bodies made from front pieces of slugs formed aberrant fruiting bodies without spores). This is, however, a cell group phenomenon and individual prespore or prestalk cells taken from these rear or front cell clumps do not show signs of acquiring the morphological appearance of the alternative cell type.

Since cellular commitment in terms of spore or stalk cells is determined by position in the slug, it is interesting to ask whether the position in the slug is further predetermined by some cellular characteristic prior to aggregation. In other words, when can the earliest signs of commitment to the differentiated fate, to a particular pattern of programmed gene expression, be seen? The first point to be made is that an early heterogeneity can be detected. So-called "prespore" and "prestalk" cells can be distinguished both by their differing numbers of cytoplasmic granules and also by differences in cell density (Takeuchi, 1969). More impressively, fluorescent antisera raised against spores will demonstrate two populations of differentially staining cells when applied to preaggregation myxamoebae (Takeuchi, 1963). It is interesting to ask whether this early indication of commitment may be related to the status of a pre-aggregation myxamoeba in terms of its cyclic AMP production, particularly since cyclic AMP secretion may well be a side product of a particular metabolic activity characteristic of a programmed prestalk cell.

It has already been emphasized that the commitment of the myxamoebae to become stalk or spore cells is not altogether inreversible and that cell clumps from dissected slugs can reform complete slugs capable of normal fruiting body production. This fact is further emphasized by some interesting observations on slugs formed from the anterior portions of other slugs (see discussion in Ashworth, 1971). In the normal slug the anterior 15% of the cells will become stalk cells, the rest spore cells. If, however, the slug is reconstituted from an isolated anterior tip from a slug, the stalk cell proportion can be as high as 80%. But, and this is surely the really exciting observation, if such slugs migrate for some time after their formation from isolated anterior tips, the further they migrate the more normal is the stalk cell/spore cell ratio. In other words, commitment and alteration in cell fate is possible and is affected by the migratory period of the slug. It is difficult not to conclude that, during this period of migration, some self recognition of a linear differentiation pattern is achieved. This has been discussed by Wolpert (1969). Although this modulation of cell fate is still not understood in *Dictyostelium*, it is obvious that it provides an excellent and simple model for the testing of theories of gradient and pattern formation. These, in their way, represent the forefront of present thinking about gene expression at the level of morphogenesis and organogenesis.

In any event, we can see from these studies on *Dictyostelium* differentiation that the myxamoebae probably do become largely, although not irrevocably, committed at a very early stage. It has been postulated that the development of the two separate populations of myxamoebae may be due to an asynchrony of growth, but it is important to stress that any early subdivision into founder cells (which attract other cells to them) and other cells (Schaffer, 1964) is in very different proportions to the pre-stalk and pre-spore segregation and the two phenomena are probably unrelated. Whatever the initial cause of the pre-spore and post-spore differentiation may be, it is apparently a simple divide which ultimately, in gene expression and cell destiny, determines whether a cell becomes a stalk cell and dies, or becomes a spore cell with the potential of further division into new myxamoebae.

The expression of these two alternative genetic programmes by aggregating myxamoebae, culminating in the spore or the stalk cell, has another interesting aspect. This is the apparent ability of the two cell types to sort out from one another, not only when they initially take up their positions within the slug, but also if the non-aggregated cells are separated on the basis of density on a dextrose gradient (Takeuchi, 1969) and then mixed together. It is by no means clear that sorting out alone determines the location and commitment of cells in the slug (Garrod and Ashworth, 1973) but at least these experiments demonstrate sorting out between cells which are, in terms of genetic expression, very similar. We can conclude that a difference of expression of only a few genes may often have effects on the cell surface which make a cell sorting out process possible or probable.

Since *Dictyostelium*, even in the slug phase, is an organism of very limited complexity, it is of interest to study the pattern of enzyme synthesis. In particular, workers in this field have sought correlations between alterations in the pattern of proteins made and the expression of the differentiated state. Of course, this sort of approach is once more a matter of studying gene expression at the level of the protein product and trying to extrapolate backwards from that to the genetic changes—never an ideal approach. Nevertheless, some interesting findings have been turned up. Probably the most interesting to date is the shape of the curve for the synthesis of individual enzymes in the differentiating amoebae. Such experiments have been made much more feasible by the isolation of axenic strains of *Dictyostelium*, permitting their growth on relatively defined substrates. For example, Garrod and Ashworth (1973) show that the synthesis of trehalase over a 50 h period is not only stepwise, but that the steps increase in size arithmetically with time. With the synthesis of some other proteins also, these authors report a response to altered growth conditions which, rather than being infinitely variable as one might expect with a completely modulated system, increases or decreases by rather simple proportions. Such suggestive observations could have an important bearing on mechanisms which regulate gene expression and, certainly, seem to suggest the utilization of new gene copies, or some such simple alteration, at a basic genetic level. It is, of course, particularly fortunate that in this system cell division and differentiation are mutually exclusive and no cell division is taking place during the assay of these enzyme levels.

In the section of this book devoted to the work on enzyme synthesis in yeast and the yeast cell cycle (Section 4B), the matter of a possible linear reading of gene sequences is discussed. There is some evidence in yeast cells that the time of onset of synthesis of enzymes in the cell cycle is related to the position of the relevant gene, early genes in a particular gene sequence being expressed early and genes further along the chromosome expressed late. Similar ideas have been frequently expressed as a means of controlling gene expression during differentiation, so that two alternatively differentiated cells arising from a common ancestral cell type might have that relationship, i.e. that differentiated cell type A has stopped its programme of changes in gene expression at one hypothetical gene, while cell type B has read through that gene (and at least theoretically has passed through the situation of being a type A cell) and has now gone on to a further differentiation which involves expressing one or more later genes. It is not easy to find experimental situations in which such ideas can be critically tested, but certain slime mould mutants seem to offer one. This has been pointed out by Francis (1969).

Some mutants which promise a test of this idea have been found in *Dictyostelium macoroides*. The MV mutant of this slime mould appears to be unable to form a fruiting stalk and lacks prestalk cells. If the cells of this mutant are mixed with wild type *D. mucoroides* cells, allowed to aggregate into a slug, and then followed in the differentiated fruiting body, all the mutant cells are found to have formed spores (Bonner, 1959). Although they have been less thoroughly investigated, two relevant types of mutants of *D. discoideum* are known, one KY 3, which appears to lack stalk cells, and the other Min 2, which lacks spores. Now, as Francis (1969) has pointed out, the existence of these two independent classes of mutant in this slime mould appear to rule out linear reading as a mechanism of the divergent differentiation of the two cell types. If one cell type, say the prespore cells, were simply a further advance in gene expression on the prestalk cells, then either defective prestalks would also mean defective prespore cells, or vice versa. If absence of prestalks but presence of prespores are found, and vice versa, as the *D. discoideum* mutants demonstrate, then it cannot be argued that one is simply an advance on the other in terms of the genes expressed.

It is worth pointing out here that studies on slime mould mutants should prove extremely rewarding since the myxamoebae are haploid. Haploidy makes a genetic study of the process of differentiation much simpler, since one of the great obstacles in understanding differentiation has always been the impossibility of carrying out a genetic analysis of differentiated cells.

2. The plasmodial slime moulds

These organisms, collectively termed the *Myxomycetes*, are plasmodial for a large part of the life cycle. Such plasmodia consist of syncitia of up to 14 cm diameter, with many millions of nuclei. The nuclei are diploid. The plasmodial or growth phase may be switched, by altered conditions of culture or environment, to a sporulation phase in which, in a highly synchronous manner, a fruiting body develops containing many discrete uninucleate haploid spores. After liberation, these spores germinate to produce uniflagellate amoeboid gametes which proceed to fuse in pairs to form biflagellate zygotes. These diploid cells will, in favourable culture conditions, grow to produce the vegetative plasmodium. The plasmodium is capable of a slow creeping movement, looking rather like a thin sheet of slime, and feeds by ingesting bacteria and decaying plant material. These plasmodia are also characterized by waves of cytoplasmic streaming and contraction which flow over the entire slime mould.

The contribution which the *Myxomycetes* can and do make to studies in gene expression is bound up with the impressive synchrony of the multinucleate plasmodium and the transition and differentiation which results in spore production.

In the experimental system used, very large synchronous plasmodia are obtained by the fusion of many small microplasmodia. Shortly after such fusion, complete synchrony of DNA synthesis obtains. It is arguable (Mitchison, 1971) that not all the plasmodial biochemistry may be as synchronous as the DNA synthetic pattern would suggest, but it is certainly probable that, after a number of cycles of DNA synthesis, the cytoplasmic cycle of synthesis is reasonably in phase, since the two processes are to some extent tied together in normal cells. The large plasmodia can now be grown axenically in liquid culture, a fact which also makes biochemical studies of the cell cycle more rigorous.

Recent reviews of differentiation studies in *Physarum* are those of Sauer (1973) and Grant (1973).

Most of the studies of enzyme synthesis in *Physarum* during the mitotic growth phase of the plasmodium have yielded essentially linear curves suggesting rather continuous synthesis throughout the cell cycle. This holds for seven out of nine enzymes followed. An exception, ribonuclease, appeared to be synthesized stepwise. These findings have been summarized and discussed by Mitchison (1971). Such a steady linear increase during the cell cycle also holds for RNA synthesis, but the protein synthesis and RNA synthesis seem to be rather loosely dependent. For example, exposure of the plasmodia to actinomycin D for longer than one generation has little effect on the pattern of protein synthesis in that period (Sauer, 1973).

Other cell cycle studies on this organism involve studies of the genetic regulation of the act of mitosis. By fusing together microplasmodia which are out of phase, it is possible to look for a superimposed synchrony of mitosis transmitted via the plasmodial cytoplasm. Although a really firm conclusion is not yet forthcoming from these experiments, one can conclude that a protein or proteins are elaborated during G_2 which have the effect of initiating or accelerating mitosis in very early G_2 nuclei.

Whether such proteins are truly specific regulators of mitosis or rather general growth stimulators is not clear. Other related experiments have involved the fragmentation of plasmodia which were in early and late G_2 and then permitting fusion between the mixed pieces in equal quantities. In such experiments, an exactly intermediate time for nuclear division could be plotted, but in fact all nuclei divided synchronously rather earlier than the calculated intermediate. This result also suggests the existence of a diffusable factor, synthesized later in G_2 which stimulates or induces mitosis.

Another aspect of the life cycle of *Physarum* which can be studied from the point of view of controlled gene expression is spherulation, the cleavage of a plasmodium into separate pieces each coated with slime and encased within a cell wall. This phenomenon is a response to unfavourable conditions such as desiccation or a very low temperature. Use of the inhibitor of protein synthesis, cycloheximide, prevents spherulation, indicating that the process is dependent on newly synthesized protein. Two such proteins have been identified, a phosphodiesterase and glutamate dehydrogenase. The manner of induction of these proteins is not clear, but in any event they are possible candidates for the class of protein so vital to studies in controlled gene expression—proteins whose presence is indicative of a transcriptional change during differentiation.

The last aspect of *Physarum* biology which we should discuss here is sporulation, the production of haploid spores following the controlled differentiation of the fruiting body. Two factors are necessary to initiate sporulation, one being illumination and the other involving the substrate (Sauer, 1973). Unlike spherulation, the process is very sensitive to actinomycin D, as well as to cycloheximide. As yet, no specific proteins have been identified whose synthesis is specific to sporulation, but several studies have been made on the pattern of RNA synthesis. There is some evidence for the synthesis of novel species of RNA during and after illumination and presumably as a preparation for sporulation. Such evidence is the existence of a new 10S RNA peak in illuminated plasmodia, a more heterogeneous RNA composition following illumination as indicated by DNA/RNA competition experiments (in such experiments illuminated-plasmodia RNA was competed with control-plasmodia RNA for hybridization to plasmodial DNA) and the actinomycin D sensitivity already alluded to (Sauer, 1973). These studies can only be regarded as suggestive, but clearly gene activity prior to sporulation is another relatively simple model for examining the relationship between altered transcription and cellular differentiation.

3. Comment

Slime moulds of both groups, cellular and plasmodial, are extremely attractive subjects for biochemical analyses of differentiation. They both display simple kinds of differentiation, can both be cultured axenically and offer some prospect of genetic analysis by selection of mutants. Surely the most promising aspects are: (a) studies on regulation of the so-called prespore and prestalk cells within dissected portions of *Dictyostelium* slugs, (b) an attempt to understand the basis for the stepped synthesis of certain enzymes in *Dictyostelium* differentiation, (c) further search for irregular enzyme synthesis during the *Physarum* cell cycle, especially after a suitable time lapse following microplasmodial fusion (an indication of linear reading of genes in terms of the time of appearance of new proteins would be particularly promising here) and (d) characterization of the specific RNA which is produced in response to illumination in the presporulation *Physarum*. Surely, the large size and nuclear synchrony of the *Physarum* plasmodium should make possible an identification of the differentiation genes concerned and perhaps of the signalling which switches them during illumination.

C. Protozoa—studies on cortical inheritance

Protozoa, as unicellular organisms, do not provide particularly useful experimental systems for the study of gene expression since the cells do not differentiate in the normal meaning of that word. However, the very fact that stocks of genetically identical individuals can be grown with relative ease permits analysis of an interesting aspect of genetic expression—the phenotypic divergence of genetically identical individuals. The most informative work has been done on two genera of ciliates, *Paramecium* and *Stentor*, and the different experimental systems will be discussed separately.

1. Cortical inheritance in *Paramecium*—cortical proteins

In 1948, Sonneborne described differences in the cortex of *Paramecium aurelia* which depended on environmental experience and not on genetic difference. The differences in the cortex of the cells were detected by raising antisera against the cell surface through injection of the *Paramecium* into a rabbit. The rabbit antisera proved lethal to the cells against which they had been prepared, but sub-lethal to some other stocks of genetically identical *Paramecium*. By testing the lethality of antisera, at least six different serotypes were detected within one stock of genetically identical individuals. The dependence of the serotype expressed on the cultural environment was demonstrated by dividing one clone of identical cells into two batches. One batch was kept in optimal conditions at 32°C with excess food, while the other batch was cultured at 20°C with insufficient food. Of the cells kept in optimal conditions, 94% became serotype B and of those kept in poor conditions 96% became H (Sonneborn, 1950). Once transformed, the *Paramecium* tend to retain their surface serotype properties and pass them on to their daughter cells.

When conjugation is permitted between genetically differing individuals which also have different surface antigens, the surface antigens of a cell may change in response to an incoming nucleus. This seems to demonstrate that the ability to synthesize a particular surface protein is dependent on the possession of an appropriate nuclear gene. The exact location of these surface proteins is not known, but the antisera raised against them immobilize the surface cilia so they may be located on the ciliary surface. A further curious aspect of these proteins follows from the fact that a cell survives brief exposure to specific antisera even when it possesses the appropriate antigen; when such cells are washed to free them from the potentially lethal antibodies, they also tend to lose the antigen. These cured cells will then develop the same or a different serotype, provided that they possess suitable nuclear genes. This evidence demonstrates that the surface proteins involved are dispensable to the cell and that their removal triggers off synthesis of new proteins to replace them, which may be an identical or different antigen. There is, then, some suggestion here of exclusive gene expression and that the product of one gene prevents expression of other nuclear genes making related but not identical molecules. But, vegetative reproduction of the cells without conjugation tends to involve retention of one pattern of gene expression, just as it does in most differentiated tissues.

Environmental effects on the pattern of gene expression are, of course, an important aspect of eukaryotic differentiation. What the *Paramecium* experiments do is to underline the process rather well and help to emphasize that a balanced pattern of gene expression which survives cell division is not exclusively a character of highly differentiated multicellular organisms.

A parallel example which we could consider in higher cell systems is the differing phenotypes of mammalian monozygotic twins. Although genetically identical, such individuals, as they live more or less independently, will encounter some differing antigens in their environment, either different strains of bacteria or viruses, or perhaps differing pollens. In response to these antigens, they will produce antibodies and, in doing so, develop in their lymphoid tissues clones of cells dedicated to the production of these antibodies. As such pairs of twins age, their differing immunological experience will, therefore, lead to differing phenotypes in their lymphoid organs, even if their more obvious facial and muscular characteristics remain apparently the same. Whether dedication to the production of one antibody molecule prevents synthesis of some others is a question beyond the scope of the present discussion, so we cannot force the parallelism between *Paramecium* clones and monozygotic twins too far.

Of course, it may well be that these surface proteins are involved as templates in the assembly of subunits. Such mechanisms are probably not uncommon in biology, as for example, in the assembly of polysaccharides such as glycogen, which require glycogen primer for polymer formation. The involvement of nuclear genes in the *Paramecium* surface antigen experiments seems to indicate that survival of template molecules for the determination of molecular architecture is not the whole story. Such problems will also be discussed in the next example of cortical inheritance.

2. Cortical inheritance in *Paramecium* and *Stentor*—cortical pattern

Various aspects of the cortex and its hereditary determination have been discussed in papers by Sonneborn (1963, 1970) and Preer (1969) on *Paramecium* and Tartar (1961, 1968) on *Stentor*.

The cell surface of these ciliates consists of a rather elaborate cortex, some one or two microns in overall thickness. A series of separate unitary structures constitute the main part of the ciliary cortex, each unit consisting of one or two kinetosomes (basal bodies) with an associated cilium, kinetodesmal fibre, parasomal sac and membranes. In some species, as in *Paramecium*, trichocysts emerge between the cortical units and appear to alternate with kinetosomes. The separate units occur in rows, giving rise to the kinetodesmal fibres which form a continuous series and occur in bundles running the length of a row of ordered units. These rows of unitary structures are referred to as kineties, and the generalized kinety pattern may be elegantly revealed by a technique involving silver staining (Corliss, 1953). In some species, such as *Stentor*, the longitudinal rows of kinetosomes and cilia which constitute the kineties alternate with stripes of pigment granules and the two should not be confused. The outermost layer of the cell surface is a cell membrane which is apparently continuous over the entire surfaces of the cilia and the other parts of the cortex. As revealed by light microscopy of the live animal as well as by silver staining, it is clear that the kinety pattern is modified to fit the shape of the organism. This is especially evident in the region of the gullet or oral cone where considerable reorganization of the cortex occurs.

The particular experiments which we are now discussing involve selection of individual organisms with visible misarrangements of the kinety pattern of the cortex. By selecting such individuals and studying the pattern of inheritance of the cortical aberration after simple fission or sexual conjugation, it has become clear that alterations in pattern are often stable for many generations, sometimes indefinitely. Actually, both *Paramecium* and *Stentor*, but particularly the former, are rather slower in readjusting the surface pattern, following damage, than are most ciliates. It is no doubt this property which has rendered these observations possible and valuable. It should also be stressed that, contrary to earlier ideas, it is now known that replication of cortical units occurs within the cortex (Dippell, 1965) and that repair and realignment of disarranged cortical order most frequently demands such replication. Moreover, cell division, which demands increase in cortical area and its subdivision between the two daughter cells, involves replication of existing cortical units and their arrangement in a pattern largely defined by the existing kinety pattern.

The cortical mutants (a mutant here being defined as an individual which is hereditarily different from the wild type) which have been studied have normally appeared spontaneously in wild type stocks, but some have been induced (Wille, 1964). Perhaps the best known of these mutants is the double animal or doublet, which has occurred in stocks of *Paramecium aurelia* and *Stentor coeruleus* and of many other ciliates (see review by Sonneborn, 1963). Although the double animal may occasionally revert to singles in culture, the stock is perpetuated more or less indefinitely by fission. The inheritance of persistence of the double animal form was studied intensively in *Paramecium* by Sonneborne and Dippell (1960); these authors were able to establish that neither rearrangement of nuclei or cytoplasm at conjugation (between doublets and normal single animals) had any influence on the inheritance of this trait. They concluded that the doublet morphology was determined by the cortex, the existing cortex prescribing the pattern of the daughter cell cortices. This conclusion has never been effectively challenged.

A second cortical mutant, described by Sonneborn (1963), is a *Paramecium* possessing a double oral segment which includes mouth, gullet and adjacent cortical structures. Again, the mutant has proved to be cortically transmitted, each daughter cell being similarly equipped with a double oral segment. Yet another cortical mutant, termed twisty, is a *Paramecium* which swims with an accentuated helical motion (Beisson and Sonneborn, 1965). Microscopical examination has revealed that a number of adjacent kineties on one side of the organism are inverted, and that the kinetosomal fibres run backwards instead of forwards in this area. Such mutants are also commonly referred to as reversed polarity mutants and gradual migration of the reversed kinetics over the cortical surface has been detected with repeated fission of such animals. The twisty mutant seems to display particularly clearly that it is the existing cortical architecture which determines the pattern of the new kinetics which are formed and that inversion of part of a kinety is a heritable trait irrespective of the nuclear or cytoplasmic endowment.

As mentioned above, other cortical mutants have also been recognized in *Stentor* and other ciliates, but the findings are essentially similar to those reported for *Paramecium*. Most *Ciliates*, including *Stentor*, are more efficient than *Paramecium* at repairing cortical pattern and restoring the original form and so, with the exception of forms like the double-animal, the *Paramecium* work is generally the most informative.

One factor has served to confuse or at least complicate thinking about cortical inheritance in ciliates and that is the claim that kinetosomes contain, or are associated with, DNA (Randall and Disbrey, 1965). The main weight of evidence now rests rather strongly against the notion that basal bodies possess DNA (Younger *et al.*, 1972), mainly through lack of evidence for DNA synthesis during basal body replication. It is probably correct to assume, therefore, that the instances of inheritance of cortical pattern discussed involve the use of existing patterns as templates for the ordered arrangement of new molecules synthesized elsewhere in the cell.

3. Comment

Gene expression in its widest sense is the production of the phenotype by the genotype. Probably the tightest aspect of gene expression is the synthesis of RNA, especially transfer and ribosomal RNA, while the determination of the primary sequence (and hence, in most cases, the three dimensional structure) of polypeptide chains is only slightly more relaxed. But from that level on the direct effects of the genes are much less pronounced. The way in which single polypeptides are arranged into large proteins with quaternary structure is probably not solely dependent on the primary structure of the polypeptides concerned. It seems possible that some large structural proteins are assembled, even in terms of their own quaternary structure, in ways which are partly dependent on existing structures functioning as architectural templates.

The inheritance of cortical pattern in ciliates is, therefore, an example of gene expression which is dependent for ultimate order on the existing cortical pattern. Presumably proteins, polysaccharides and other molecules, synthesized elsewhere in the cell, are shipped to the cell surface to be assembled to the design of the existing cortex. The ciliate cortex, therefore, takes its place along with such polysaccharides as glycogen, and probably the cell walls of many bacteria (see discussion in Maclean, 1975) as a structure which assembles precast molecules in an order dictated by the existing structure. The control of gene expression is not, therefore, a unilateral and one way hierarchy and heredity is dependent on other templates besides DNA.

D. *Acetabularia*

This marine alga is one of the most famous research organisms in the world, at least in laboratories concerned with cytology and genetics. The reason for its fame coincides with our interest in it in this book—namely that it consists of a single giant cell and exchange of nuclei and cytoplasm between separate cells can be accomplished with comparative ease by mechanical grafting.

Acetabularia belongs to the class *Dasycladaceae* and, when mature, measures up to five cm in length. The form of the plant is that of a long stalk attached to a substrate by a group of rhizoids (see Fig. 17). In the young alga the stalk supports whorls of small branches at or near the tip; when approaching maturity many of these hair like branches fall off and a terminal cap or umbrella is formed. This cap is quite large and may measure one cm in diameter. The cap is a reproductive organ, since it becomes subdivided into radial chambers, or rays, which are eventually packed with cysts. A number of different species of *Acetabularia* occur, of which *A. mediterranea*, *A. crenulata*, *A. peniculus* and *A. cliftonii* are the best known. The latter two are probably synonyms for the same species. Our description of stalk and cap best fits the first species. One of the very favourable aspects of this organism is that the form of the stalk and cap vary considerably in the differing species. Such differences have permitted experimenters to examine the effect of inter-specific exchange of nuclei on the eventual morphology of the cap.

Acetabularia is a common alga along the Mediterranean shore line, the stalk and cap frequently being encrusted with calcium carbonate in nature. The rhizoids normally attach to rocks, pebbles or shells. Its reproduction involves breakdown of the caps with release of the cysts. These cysts then rupture to release numbers of motile flagellated zoospores which, depending on the prevailing environmental conditions and the species, may either grow on vegetatively into a new alga on settling, or alternatively fuse with other zoospores of an opposite sex to form a zygote which then grows on into a new alga. The structure and natural history of this interesting organism is fully discussed by Puiseux-Dao (1970). This alga can be cultured in the laboratory with reasonable ease and its successful maintenance under experimental conditions has been pioneered by Hammerling, whose numerous papers are listed in Puiseux-Dao (1970). In culture, the early growth is often slow and cap formation normally occurs only after about six months of growth. The time taken for zoospores to form a visible green sphere may be as long as the second stage in which the cell dramatically enlarges to form rhizoids, branches and ultimately a fruiting cap. If conditions become unfavourable

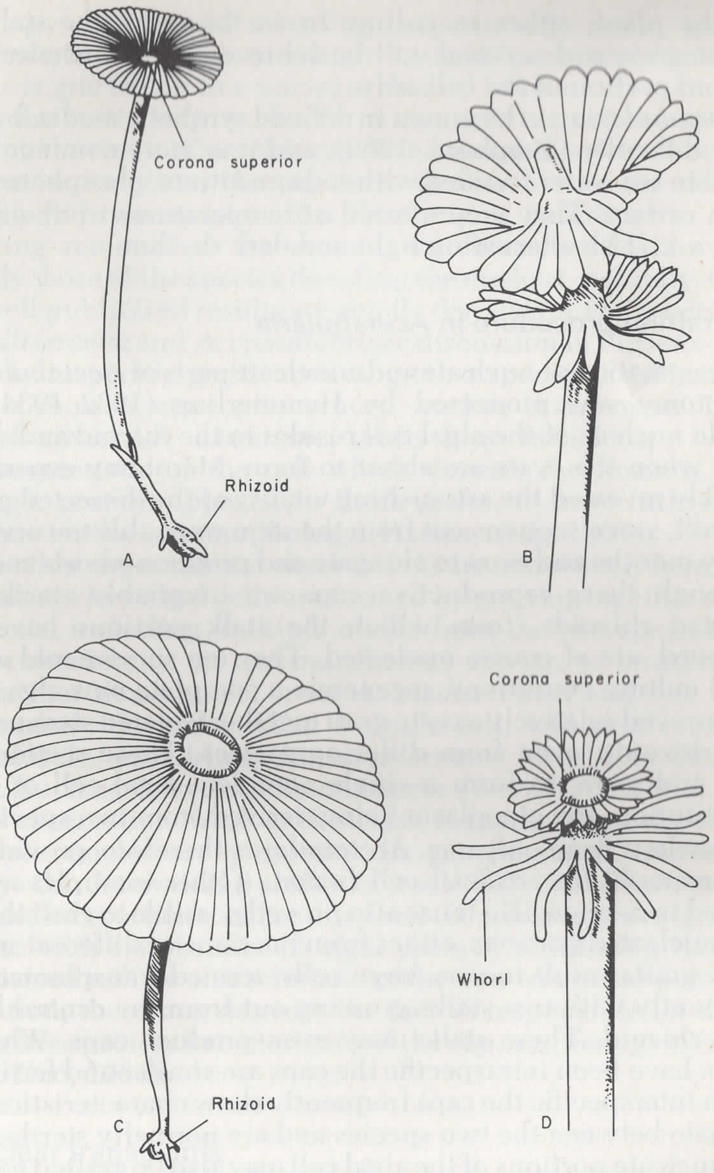


FIG. 17. Regeneration of caps in *Acetabularia*. A and C are young and adult caps regenerated by nucleated rhizoids of *A. meditteranea*. B and D are caps and whorls regenerated on basal portions of *A. crenulata*. These drawings demonstrate the regenerating potential of the algal rhizoid and the characteristic cap morphology of the two species (from "Growth, Development and Pattern" by N. J. Berrill. W. H. Freeman and Co. C 1961; after Hammerling).

for the plant, either in culture or in the wild, the stalk may disintegrate and survival will be achieved by persistence of the rhizoid portion of the cell only.

Acetabularia can be grown in defined synthetic media, but only with difficulty (Shephard, 1969), and it is more commonly cultured in sea water fortified with sodium nitrate, phosphate and an earth extract. They are cultured at temperatures of about 20°C with a 12/12 h alternating light and dark rhythm.

1. Grafting procedure in *Acetabularia*

The separation of nucleate and anucleate parts of *Acetabularia* by merotomy was pioneered by Hammerling (1932, 1934). The single nucleus of the algal cell resides in the rhizoid and divides only when the cysts are about to form. Merotomy experiments quickly revealed the astonishing vitality of the dissected parts of the cell, since sections cut from the stem were able to survive for many months and even to elongate and produce whorls and caps, although these reproductive caps are invariably sterile. The isolated rhizoids, from which the stalk portions have been removed, are of course nucleated. They too survive and will, in good culture conditions, regenerate a complete new alga. It has also proved relatively easy to graft together isolated stem portions and rhizoids, even from differing species. These sections will fuse and grow to form a single complete algal cell of mixed constitution, the cytoplasm being derived from two species and the nucleus from only one. *Acetabularia*, therefore, provided the first material for artificial cell fusion. (Other examples are discussed in Section 4E.) It has also proved possible to graft together two nucleated portions, either from the same or different species. Such grafts result in very large cells, termed transplantat, most frequently with two stalks growing out from the central binucleate rhizoid. These stalks may even produce caps. When the grafts have been intraspecific the caps are similar and fertile, but when interspecific the caps frequently show characteristics intermediate between the two species and are normally sterile.

Anucleate portions of the algal cell may also be grafted together and will survive for some months. If the grafts are, in this case, intraspecific and, particularly, if the grafted portions include a stalk apex, then a cap typical of that species may form. If the grafted anucleate portions are from differing species, however, the cap will be intermediate in form and sterile.

Perhaps the most revealing experiments with grafted *Acetabularia* are those in which an anucleate stem from one species is grafted on to a nucleate rhizoid from another. In such grafts, the initial cap is of a form characteristic of the species donating the stem. When this cap is severed and another allowed to grow in its place, the cap form is now often intermediate between the two species. If a third cap is permitted to grow following removal of the second, its characteristics are now strongly those of the species donating the nucleus. Actually, these now well publicised results are chiefly derived for grafts between *A. mediterranea* and *A. crenulata* (see discussion in Puiseux-Dao, 1970). Intraspecific grafts involving other species combinations tend to show a more pronounced nuclear dominance. Thus, *A. mediterranea-A. wettsteinei* grafts form initial caps which are characteristic of the cell which donates the nucleus. The anucleate portion appears, in these grafts, to have little or no influence on the cap morphology (Hammerling, 1953).

Two other experimental observations are noteworthy here. The first is that when algal cells are formed by grafting together many fragments, including multiple nuclei from the two species, the nuclear dominance on cap morphology appears to be additive. That is that three nuclei from *A. mediterranea* and one from *A. crenulata*, if combined in a single cell, will produce caps more closely resembling the *mediterranea* form than the *crenulata*. Such caps are, in general, sterile (Werz, 1955, 1965). The second observation involves the use of X-irradiation to destroy or inactivate nuclei or cytoplasm. In general, such irradiation destroys the determining ability of nucleus or cytoplasm, so that if irradiated portions either nucleate or anucleate are grafted together with non-irradiated algal parts of a differing species, caps formed will be characteristic of the non-irradiated portions. Irradiation, therefore, destroys the factors responsible for determining the species characteristics of the cap morphology (Six and Puisex-Dao, 1961).

2. Nuclear transplants

As well as the merotomy and grafting of sections of *Acetabularia*, some experiments have been undertaken on isolation of nuclei, relatively free from cytoplasm, and their insertion into other portions of *Acetabularia*. Clearly, such experiments are desirable in order to ensure that the strong influence of the rhizoid portions on cap morphology are truly a result of the residence of the nucleus in that section. In these experiments the base of an alga containing the nucleus is placed in a solution of buffered sucrose at 4°C and the nucleus squeezed out by gentle pressure. Nuclei will survive in this medium for only a few minutes, but can be injected, having been taken up into a micropipette, into other already anucleate basal segments. Some normal and fertile alga have resulted from such nuclear transplants. These experiments are still rather few in number and most have been intraspecific grafts, but they do tend to support the idea that the nucleus in the basal rhizoid is the source of the morphogenetic determinants in cap formation (Hammerling, 1953).

(a) *Some conclusions from the merotomy and nuclear transplant experiments*

The experiments discussed above provide a basis for very sound conclusions on the relationship of nucleus and cytoplasm in these cells. They are, in brief:

1. Factors determining cap morphology exist in the cytoplasm of the algal cell and are very long lived.
2. Such factors can be destroyed or inactivated by X-irradiation.
3. The long term source of the cap-determining factors is in the basal rhizoid and almost certainly in the nucleus.
4. In cells with many nuclei, the cap form will tend to be that of the species donating the greater number of nuclei.
5. Anucleate stem portions are able to form caps many weeks after the removal of the nucleus.

A useful discussion of the *Acetabularia* grafting experiments and their interpretation will be found in Harris (1974).

3. Biochemical aspects of the *Acetabularia* experiments

(a) *General*

In recent years, the growth and morphogenesis of the *Acetabularia* cell have been studied in terms of their biochemistry and assays developed for the detection of particular molecular species (see discussions in Puiseux-Dao, 1970; Harris, 1974). One aspect which has been studied in great detail is cap formation. This event is now known to involve net synthesis of protein and the synthesis of specific polysaccharides and enzymes. Some polysaccharides present in the cap are not found elsewhere in the cell and cap formation demands not only production of these sugars but also of the enzymes necessary for their synthesis.

One complication to the *Acetabularia* work should be discussed here. This cell is a plant and possesses chloroplasts, each of which is endowed with its own DNA genome. Moreover, as with all eukaryotic cells, mitochondria also occur in the cell and possess their own DNA. It is obviously crucial to eliminate the possibility that much of the morphological programming already discussed is mediated by non-nuclear DNA. Attempts to influence cap morphology by transplantation of chloroplasts or crude cytoplasm indicate that the source of the determining factors is not in either of these fractions (Schweiger, 1969). It is also possible that the allegedly long life span of the molecules determining cap morphology is illusory and that they are capable of independent production in the cytoplasm, at least for a time. Such possibilities might involve production of multiple free copies of the relevant genes and their release into the cytoplasm, i.e. gene amplification as found with the ribosomal cistrons of *Xenopus* during the earliest stages of development. Alternatively, the discovery of reverse transcriptase enzymes in some RNA viruses raises the possibility that cytoplasmic gene copies might be transcribed in the cytoplasm from RNA. All that can be stated with certainty at the moment is that such mechanisms appear to be confined to very specialized biological situations and that there is absolutely no evidence from the *Acetabularia* experiments which would suggest their relevance to that cell. Instead, a considerable body of the biochemical evidence argues for long lived but non-replicating RNA templates in the cytoplasm of *Acetabularia* being responsible for the cytoplasmic influence on cap morphology.

(b) *Protein synthesis*

It is clearly of interest to study the effects of grafting and particularly of enucleation on the synthesis of individual proteins. But, great care must be exercised in the interpretation of the results, since proteins synthesized within the mitochondria and chloroplasts will continue to be served by DNA templates after enucleation. Synthesis within the chloroplasts tends to be identifiable by its tendency to be stimulated in the light. Two enzymes which are not apparently localized within a cellular organelle are aldolase and glucose-1-phosphate and in each of these examples, synthesis was found to persist for ten days (Baltus, 1959) and 20 days (Clauss, 1959) respectively. When an enzyme which is associated with the chloroplasts is assayed (the assumption being that such proteins may be coded for by the chloroplast genome; this assumption is not always justifiable, since some mitochondrial and chloroplast proteins are known to be coded for by nuclear genes) then enucleation has little effect; indeed, increased synthesis has been observed in the case of two isoenzymes of acid phosphatase (Triplett *et al.*, 1965). The fact that the decline or termination of the synthesis of specific proteins in anucleate *Acetabularia* fragments follows directly from the absence of the nucleus is borne out by the resumption of such synthesis following addition of a nucleus (Richter, 1959).

A favoured way of studying the nuclear dependence of protein synthesis has been the use of specific chemical inhibitors such as actinomycin D. Some of these substances are less specific in their action than is sometimes suggested and, of course, many will affect the intra-mitochondrial and intra-chloroplast synthesis as well. The most clear cut evidence with actinomycin D is that addition of the drug to very small plants, even those retaining a nucleus, will prevent further morphogenesis and will certainly stop cap formation. On the other hand, exposure of large pieces of anucleate stalk to this chemical has little effect and cap formation will continue in its presence. It seems certain that these experiments provide further evidence for the lack of DNA dependence of much of the protein synthesis of the mature *Acetabularia* cell.

Cap formation by anucleate stem portions of this alga can also be shown to be dependent on chloroplast function. If DNA synthesis is prevented in the anucleate fragments by the addition of hydroxyurea or ethidium bromide, then cap formation is prevented (see discussion in Puiseux-Dao, 1970). Such results point to the significance of the mitochondria and chloroplasts in maintaining growth and development in the cell and again stress the general importance of the cytoplasm in cell differentiation.

Two other interesting observations on protein synthesis derive from *Acetabularia* experiments. The first is that synthesis of different enzymes need not occur simultaneously in the cytoplasm and that some cytoplasmic regulation of translation is evident. This follows from observation by Spencer and Harris (1964) on the synthesis of different phosphatases in nucleate and anucleate fragments. A second observation points to the same conclusion. It involves the removal of stalks, or at least of stalk apices (the tip of the stalk appears to act as a reservoir of the cap-determining factors in *Acetabularia*) from algal cells, followed by incubation of the nucleated fragments in solution containing Actinomycin D. This treatment is given either immediately after apex removal, or at a time following a delay of one, two or three days up to a 15 day delay (Zetsche, 1964). Such experiments show that cap formation will follow at least in a percentage of the exposed fragments, if only three days is allowed to elapse before Actinomycin D treatment, despite the continuation of the exposure up to 47 days from apex removal. When the inhibitor was removed, stalk formation and eventual cap formation proceeded in many cases. This experiment also seems to suggest that in three days the nucleus released all of the morphogenetic factors and that the extended time scale of the utilization of these factors in forming stalks and caps was essentially a cytoplasmic event.

(c) *RNA synthesis*

Aside from the special phenomena of reverse transcription (see, for example, Bobrow *et al.*, 1972) and gene amplification (Brown and Dawid, 1968) the DNA of the nucleus normally determines the amino acid sequence of the proteins made in the cell cytoplasm via RNA intermediates. The finding that novel proteins appear in anucleate fractions of *Acetabularia*, therefore, focuses interest in the stability of the RNA intermediates. This algal cell is, therefore, the chief (though not the only) source of strong evidence about the life span of messenger RNA molecules in eukaryotic cells. RNA synthesis and characterization has been accomplished in this cell by means of incorporation studies with labelled RNA precursors, colorimetric determination of RNA and electrophoresis and centrifugation of the cytoplasmic RNA. Much of this work has been done in the laboratory of Professor J. Brachet (see Brachet, 1963, 1965).

Despite early reports to the contrary, it is now clear that considerable RNA synthesis occurs in anucleate fragments of *Acetabularia*. Unless evidence can be provided for gene amplification or reverse transcription, however, it must be assumed that all of this RNA synthesis is mitochondrial or chloroplast in origin. There is, therefore, no good evidence for RNA synthesis providing a continuing source of cellular RNA. This being so, it can be asked to what extent cap formation actually requires messenger RNA. The answer, already given, is that considerable net protein synthesis occurs during cap formation in anucleate *Acetabularia* stems, including elaboration of some apparently new enzymes. As mentioned above, it can also be observed that Actinomycin D, which inhibits RNA synthesis on DNA templates, has little effect on cap formation either in mature whole cells or large anucleate stem fractions (Zetsche, 1964).

4. The autonomy of the chloroplasts

Since chloroplasts are, like mitochondria, micro genetic systems resident within the eukaryotic cell, the anucleate *Acetabularia* cell is obviously excellent material in which to examine their dependence or independence from the nucleus. This aspect of *Acetabularia* biology also receives detailed discussion in Puiseux-Dao (1970). It appears that plastid multiplication continues normally in anucleate fragments for about seven days, the plastid DNA doubling time being about 5–7 days (Shephard, 1965). Since it is also known that some of the genes for chloroplast proteins are located exclusively in the nuclear genome (Apel and Schweiger, 1972), it follows that chloroplast replication without a nucleus must soon deplete the stores of these proteins. In fact, the size of the chloroplast genome, although considerably greater than the mitochondrial one, is, nevertheless, clearly insufficient to code for all of its own repertoire of proteins (Schweiger, 1969). The possibility of a bacterial origin for the chloroplast is also suggested by the sensitivity of the *Acetabularia* chloroplast to the action of chloramphenicol (Puiseux-Dao, 1970), an antibiotic with a relatively specific action on the bacterial ribosome and little effect on eukaryotic cytoplasmic ribosomes.

5. Comment

It must be clear that the easy dissection of this enormous cell into nucleate and anucleate fragments and its ready recovery and fusion of joined fragments have made it a most valuable biological research material. The experiments on this cell leave little room for doubt about the persistence of cytoplasmic messenger RNA for many weeks, together with ribosomal and transfer RNA molecules. In addition, these experiments also tell us that some messenger RNA may be stored without immediate translation. The differing cap morphologies of related species of *Acetabularia* have also permitted the formation of fused cells of hybrid origin and in these situations the nucleus will ultimately determine the form of the cap, but not necessarily of the first caps appearing on such hybrid cells.

If messenger RNA is stable in most eukaryotic cells for weeks or months and if it can be regularly sequestered for future translation, then much of the onus for governing genetic expression on a short time scale falls on the cytoplasm. A similar conclusion comes from Gurdon's experiments on message injection discussed in Section 4E. It is a pity that the early experiments on messenger RNA involved prokaryotes and yielded evidence suggesting a very short life span for the molecule. But, we must equally well be cautious about moving now to the other extreme by assuming that all eukaryotic messenger RNAs are very long lived. Actinomycin D rapidly affects specific protein synthesis in some differentiated eukaryotic cells (Maclean and Madgwick, 1973) and, very probably, the lives of messenger RNA will prove to be very variable from one mRNA type to another and one tissue to another.

The partial genetic autonomy of mitochondria and chloroplasts is now well established, but is particularly well demonstrated in work on *Acetabularia.* It is desirable to study controlled genetic expression within the mitochondrial and chloroplast genome, but in order to do so a multicellular organism with differentiated cells and cellular organelles might be the best choice. If these organelles have carried over prokaryotic genetic tendencies with them, then it might be interesting to look at the life spans of mitochondrial and chloroplast messenger RNA. Unlike that of the eukaryotic cell cytoplasm, it may turn out to be very short lived.

E. *Hydra*

Hydra has attracted the attention of students of morphogenesis for many years, chiefly because of its relatively simple form and morphological plasticity. Our interest in it in this book is rather precise, namely that it may be the only organism in which overtly differentiated cells can alter their form and change to another kind of differentiated cell without an intervening mitosis. There is then, altered gene expression without cell division. It has already been noted in the discussion of imaginal discs, slime moulds and other systems that differentiation tends to involve tightly restricted programmes and that the end product of this process is normally a cell in an exceedingly stable state. So-called dedifferentiation is rare and when it does occur, as say in the growth of a carrot plant from a carrot phloem cell, it normally involves cell division. Hydra is, therefore, a most interesting and important exception to an almost universal rule. In order to assess and appreciate this phenomenon, it will be necessary to discuss something of the organisation and biology of *Hydra*.

The cytology and cell differentiation of *Hydra* is authoritatively discussed by Burnett (1968, 1973). Burnett lists ten distinct cell types in this organism, including the so-called interstitial cells which are categorized by the absence of morphological specialization. Within some of the basic ten cell types, minor variants may also be found. The ten types of differentiated cell types in *Hydra* are nerve cells, cnidoblast cells, gastrodermis mucous cells, gastrodermis gland cells, interstitial cells, epithelio-muscular cells, digestive cells, basal disc mucous cells, sperm cells and egg cells. In terms of general anatomy, *Hydra* comprises a basal disc, which is attached to the substratum, and a short solid peduncle, which subtends the long hollow trunk or gastric region. Surrounding the upper end of the gastric region is a circle of about six long tentacles and, rising between the tentacles, a cone shaped hypostome with the mouth at the top. The body wall of the trunk or gastric region is basically a two layered structure, with an outer epidermal layer of epithelio muscular cells and cnidoblast cells, with some nerve cells between, and an inner gastrodermis comprising the mucous, gland and digestive cells. Cnidoblasts are also particularly abundant in the tentacles, containing as they do the nematocysts which play a role in anchorage, defence and the capture of prey. In some species of *Hydra*, a symbiotic alga, *Chlorella*, is to be found in the gastrodermal cells and it is these algae which give many *Hydra* their bright green colour. Interstitial cells are commonly interspersed amongst the other cell types in epidermis and gastrodermis, but they are rare or absent in the hypostome and tentacles.

The interstitial cells are the stem cells for production of nerve cells, cnidoblast cells, sperm cells and ova and, perhaps, of other cell types also (Burnett, 1968). Between the epidermal and gastrodermal layer there is a mucous sheet termed the mesoglea, or middle jelly. Although this material had been viewed earlier as some sort of cement, the independent movement of whole sheets of epidermal and gastrodermal cells makes it plain that it is a

substrate which permits easy cell movement over and perhaps through it (Campbell, 1967). See also discussion in Webster (1971). Movement and differentiation of the interstitial cells of *Hydra*, interesting though it is, is not relevant to our present subject. The more pertinent topic of differentiation of one cell type from another already differentiated cell is achieved by the artificial explantation of pieces of *Hydra* tissue.

1. Regeneration in explanted *Hydra* tissue

Explants of *Hydra* tissue, taken from the gastrodermis region, will not survive in media normally used for *Hydra* culture, including pond water. However, they will survive in other aqueous media with altered ionic content (Burnett, 1968, 1973), although complete *Hydra* will not tolerate these altered media. As the explant grows, it is transferred through media of diminishing ionic strength until it can finally be placed without injury in the normal *Hydra* culture water. Such explants can be very small and yet successfully regenerate an entire new animal and it is known that explants containing only two cell types, digestive and gland cells, can on occasions regenerate a complete *Hydra* (Burnett, 1966 and Davis, 1973). Most commonly, this involves apparent dedifferentiation of gland cells at the periphery of the explant into cells structurally indistinguishable from interstitial cells. This change is then followed by rapid division of the new interstitial cells into cnidoblast, nerve and other cells including sperm cells. It must be immediately clear to the reader that such observations cannot easily exclude the possibility that the regeneration occurred from hidden interstitial cells in the explant and it does seem particularly important that such a possibility should be excluded.

Davis (1973) discusses this problem at some length and provides descriptive and electronmicroscopic evidence for the change from gland cell to interstitial cell and interstitial cell via cell division, to many other cell types. He describes the reduction of endoplasmic reticulum and release of secretory droplets as the gland cells take on the appearance of interstitial cells, but does add that some of the gland cell structures may not be wholly lost and that some of the secretory droplets, so characteristic of the gland cell, may still be found in the redifferentiating interstitial cell. Davis' evidence is very convincing so far as it goes. A precise definition of an interstitial cell or neoblast (Burnett, 1973) is not possible, since it tends to be defined by its lack of distinctive morphology. It is, therefore, impossible to exclude the possibility that the interstitial cells of the normal *Hydra*, apparently acting as generalized stem cells, may not be identical to the interstitial cell which forms a temporary intermediate in the transformation of a gland cell to, say, a cnidoblast.

The second and more interesting regeneration described (Burnett, 1973) involves digestive cells on the periphery of a gastrodermal explant. These cells normally contain algal cells within them. Following explantation, the algae within the digestive cells disappeared, the cells developed longitudinal muscle fibres and began to secrete mucous. Normally, both of these characters may be displayed by gastrodermal cells but the muscle fibres and mucous secretion of the gastrodermal cell are both readily distinguishable from those characteristic of epidermal cells. What Burnett (1973) is saying is that the gastrodermal cells took on the characteristics of an epidermal cell. This transformation, he states, is accomplished without cell division. Again this observation is fascinating for the student of differentiation and is especially relevant to our topic, since it presumably involves a change in genetic programming.

Since the epitheliomuscular cells of the epidermis are normally capable of division, the newly formed epidermal cell will follow its development from the gastrodermal cell, proceed to divide and produce other epitheliomuscular cells. It is a pity that such an important and interesting observation cannot be recorded on film or at least expressed in terms which can be more readily verified by the serious student. Obviously, these experiments in *Hydra* are cleverly devised and expertly executed and the authors quoted have no doubt striven to present their information as accurately as possible.

Assuming for the moment that the observations and their interpretation are authentic and correct, it does mean that at least in some cases, alteration of the terminal genetic programme of a cell need not involve cell division. Now, it might be that the two differentiated states involved are not normally terminal, but that one is normally sub-terminal, so that the transformation would be tantamount to the development of an erythroblast into a reticulocyte, a step which in at least some organisms need not involve cell division. Such an explanation, although it still seems possible, is not rendered more likely by the apparent separateness of the layers of inside and outside cells in *Hydra*. It is not certain whether any cells are normally capable of making the passage between the two sides.

The role of the neoblast or interstitial cell in *Hydra* has received further attention from Burnett and his colleagues. A fascinating result was obtained from experiments designed to accomplish regeneration of a complete *Hydra* from an isolated basal disc. Interest in the basal disc was aroused by its apparent lack of interstitial cells, gland cells and cnidoblast cells. Although many of the basal discs which had been dissected away from complete *Hydras* did not survive, a few did and three were found to regenerate considerable parts of the complete animal. These regenerated forms had many interesting aberrations, including absence of mucous cells and cnidoblasts. But much the most interesting observation on these regenerates was the complete absence of interstitial cells. *Hydras* regenerated from groups of epitheliomuscular cells of the epidermis are also found to lack interstitial cells. We have already commented, however, that normally when animals are regenerated from some cell types, for example gland cells and digestive cells of the gastrodermis, intermediate interstitial cells are formed from these differentiated cells. These interstitial cells will then proceed to regenerate other cell types.

On the other hand, it has been observed that *Hydras*, treated with X irradiation or nitrogen mustard, although capable of limited regeneration, invariably die. The treated animals are able to survive for several weeks following exposure, but are found to lack interstitial cells completely (Diehl and Burnett, 1964).

It, therefore, appears that (a) interstitial cells are a necessary source of stem cells for the production of differentiated cell types and (b) that some of these differentiated cells, but not others, can provide a new stock of interstitial cells by a process of de-differentiation.

Hydra appears to be an interesting organism in which the very process of differentiation is still in an early stage of evolutionary development. Thus, the programme of gene expression characteristic of any one cell type is apparently less stable than in many higher organisms. The developmental process may often be renewed so that gland cells can revert to the more totipotent interstitial cells, but the epitheliomuscular cells cannot. These epidermal cells will proceed further along the differentiating pathway to produce mucous cells, zymogen cells, digestive cells and nerve cells, but most frequently without any intervening cell division. The differentiated state of these cells is rather unstable and can easily be moved into a differently programmed state of differentiation.

2. Comment

Hydra is a fascinating organism in terms of the relationship between differentiation and the selection of a genetic programme. A relatively undifferentiated stem cell, the interstitial cell, seems necessary for the continued production of some of the ten types of differentiated cells of *Hydra.* Some of these cell types can revert to the stem cell morphology and redifferentiate into another distinct cell type. Other cells in *Hydra* do not retain this plasticity. On the face of it, *Hydra* is an organism in which the process of differentiation is itself developing and has not yet reached the state of irreversible genetic programming characteristic of most eukaryotic organisms. Of course, the stem cells of, say, the erythropoietic system are themselves a necessary compensation for the extreme loss of versatility found in the erythroid tissue of higher organisms. The higher eukaryotes cannot normally boast of a system in which a highly differentiated cell can return to the stem cell state, or revert to another cell type without an intervening cell division. Whole carrot plants have been grown from carrot phloem cells but, in this case, the extent of the differentiation of the competent cell was not clear. It is obvious that many of the difficulties of tissue repair or replacement found in the higher eukaryotes result from the extreme stability of the genetic programme expressed in many differentiated cells. Quite probably, this is an inevitable consequence of a complex system of differentiated cells. If cells are to be highly specialized, they cannot afford to deviate from their expected path of gene expression. Some deviation might be disastrous—e.g. the production of an unwanted degradative enzyme. The remarkable rarity of diseases of faulty differentiation (perhaps the human disease of xeroderma pigmentosum may be one, since affected individuals lack an endonuclease enzyme normally involved in repair of DNA after u.v. irradition) points to the effectiveness of the tight genetic programming of the higher eukaryotes.

The evidence from the *Hydra* experiments is somewhat less watertight than one would like. A search for biochemical markers amongst *Hydra* differentiated cells would surely be possible. Even antisera raised against epidermis and gastrodermis might, when precipitated out against the opposite antigen, leave some specific antibodies. These, if conjugated with fluorescent dyes, should make much more reliable cell recognition assays than the appearance of general morphology viewed in the light or electron microscope. It seems particularly necessary to clarify the identity of the interstitial cell and so ensure that all cells dubbed with this name are equivalent in terms of their gene expression. If all attempts at biochemical marking prove non-feasible, then time lapse cinephotography of one cell actually changing its morphology might be possible—it is always difficult for someone outside a particular experimental system to guess what would be experimentally possible in that system. The *Hydra* system is a useful and important one in terms of our understanding the stability of the differentiated state and, with some refinement of technique, could provide more definite information in the future.

F. The imaginal discs in *Drosophila*

It is not easy to find a biological system in which the role of gene expression during differentiation can be easily explored. It is particularly difficult to find ways of examining the genes which actually control differentiation. The best biological studies are almost always accomplished by finding a system which provides answers to problems naturally, so that the experimenter utilizes a situation in which his experiment is carried out as part of the normal biology of the organism. The imaginal discs of insects, especially of *Drosophila,* seem to provide an unrivalled means of studying the relationship between differentiation and gene control.

Imaginal discs consist of small nests of cells which are committed in early development to become the source of the adult integument. When the discs arise in the larva, they appear as groups of undifferentiated cells, and they can persist in that state for long periods of time. However, each disc is committed to a particular fate in terms of the part of the integument which it will form, this fate being realized only after metamorphosis of the organism and differentiation of the discs into the integumental areas. In *Drosophila,* most of the surface of the adult fly arises from disc cells and the structures associated with the surface result from the differentiation of the different imaginal discs. There is evidence that part of the abdomen may not arise from true imaginal discs, at least in the blowfly, *Calliphora* (Pearson, 1972).

Drosophila has ten pairs of discs plus a single genital disc, each disc having a characteristic location in the larva. The size and shape of different discs is also characteristic, most consisting of a few hundred or a few thousand cells. The adult integument is, therefore, like a jig-saw puzzle which is assembled from different

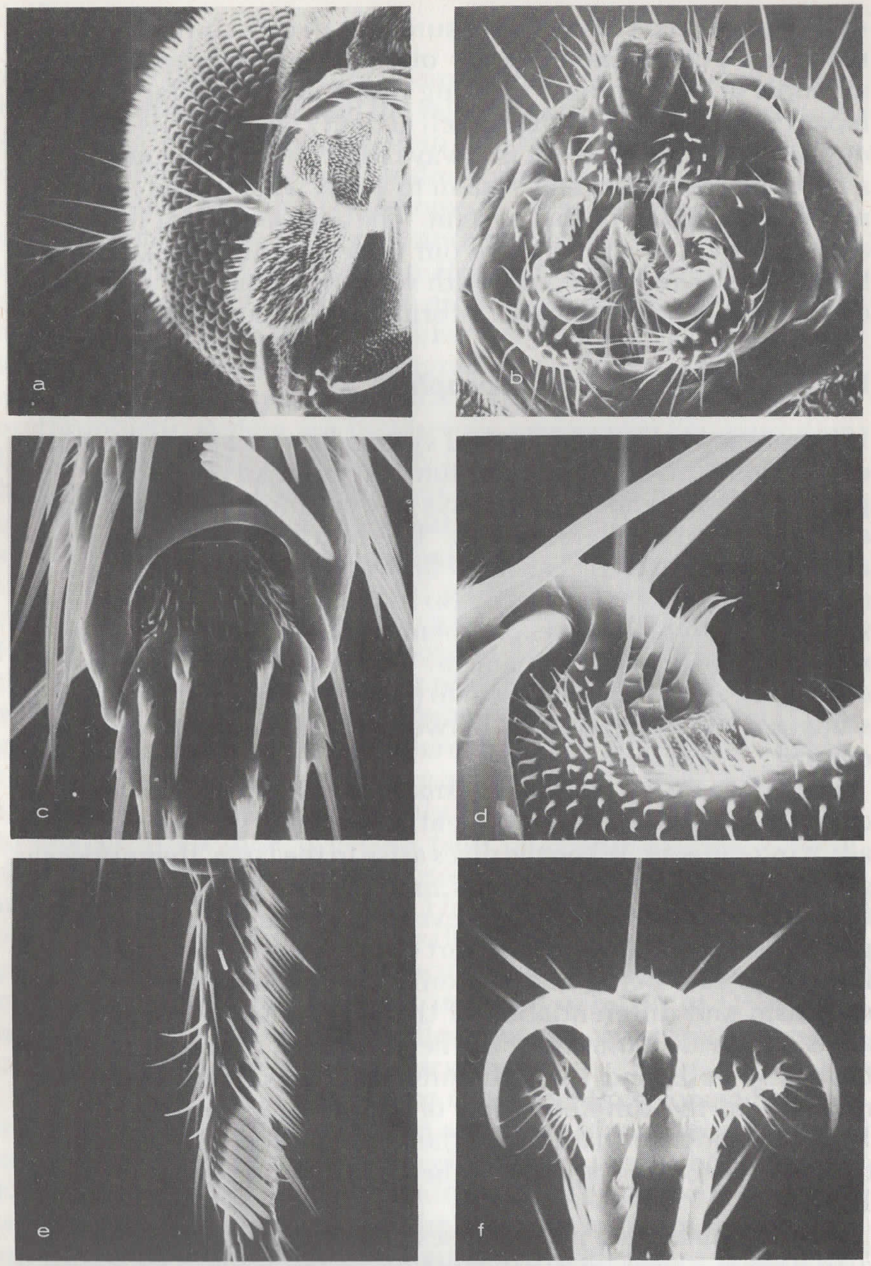


FIG. 18. Scanning electron micrographs of various imaginal disc derivatives in *Drosophila*: (a) eye and antenna, (b) male genitalia, (c) tibia-basitersus joint, (d) group of sensilla trichodea on the leg, (e) basitarsus with sex comb and (f) tarsal claw (from photographs kindly provided by Dr. P. Bryant; see Bryant 1973).

pieces, each piece the product of one disc. For example separate discs produces eyes, legs, wings, halteres and genitalia, while the head capsule is the product of three pairs of discs, including the eye discs (see Fig. 18).

Histologically the discs are very simple, each consisting of a single layer of epithelial cells arranged in the form of a sac surrounding a lumen, the sac being attached to the epidermis of the larva by a thin stalk. At metamorphosis, the small hollow sac everts, converting the folded epithelium of the disc into the extended surface of the integument. Individual disc cells are fairly typical of undifferentiated embryonic tissue, with large nuclei and little endoplasmic reticulum. However, despite appearances, the disc cells are already tightly programmed to develop along particular paths. If an eye disc is removed from a larva, the resulting adult will lack an eye; if the eye disc is transplanted to the body cavity of another larva, the disc will proceed to form an eye in the new host following metamorphosis. Imaginal discs, therefore, provide an excellent model system for the study of the genetic control of cellular determination and differentiation. A recent review by Postlethwait and Schneiderman (1973) discusses the developmental genetics of *Drosophila* imaginal discs at some length, and also lists a number of the special advantages offered by this experimental system. They are as follows:

1. Since each disc consists of a layer of cells and each produces a portion of integument, complete with numerous identifiable markers such as bristles, the development and fate of individual cells can be followed and plotted.
2. Microsurgery and culture in non-parental hosts is relatively easy, since the discs can be sucked up into micropipettes and simply deposited by injection into the abdomen of another larva or adult fly. Repeated transplantation and culture in the abdomens of adult flies prevents differentiation of the disc cells for as long as required. Reimplantation into a larva, followed by metamorphosis, then permits the realization of the developmental potential of the disc as it goes to form its section of integument.
3. Two distinct processes are widely separated in imaginal discs—determination and differentiation. This is, perhaps, their greatest advantage and it is doubtful if any other biological system can rival them in this regard. When a disc cell

passes on to all its progeny the commitment to form, say, leg integument, then that cell is said to be determined, though it may as yet show none of the overt signs of its developmental commitment. However, if a clone of cells demonstrates a variety of different fates after metamorphosis, the parent cell is looked on as relatively undetermined.

4. The establishment of spatial pattern is an important ingredient of morphogenesis and in many ways, appears to be under loose genetic control. By following the fate of individual cells within a disc the control of the pattern formation can be studied rather easily. Moreover, it can be distinguished from the more basic process of determination. Bryant (1973) has proposed the term specification to describe the former process.
5. Genetic markers can be produced in the form of X-ray-induced chromosomal abnormalities. Discs may be found which are mosaic for the marker, making the study of individual cell development and commitment easier.
6. *Drosophila* is genetically well worked and many mutants are available. Analysis of known mutants reveals that those involving the imaginal discs (and of course many do) fall into two distinct classes—some involving determination and others only specification. Analysis of the biology of these mutants is highly informative in terms of understanding these two processes.

The experiments on imaginal discs which have a bearing on the control of gene expression are very numerous and only some selected examples can be discussed here. We will do so under headings which denote distinct groups of experiments.

1. The onset of developmental commitment

One important question in embryology is to ask when, during development, a cell or nucleus becomes committed to its fate. This question can usefully be asked of the imaginal disc situation. The answer is that, in general, disc commitment is not traceable back to the insect egg, i.e. there is no evidence for determinants being active or identifiable in the egg, which will destine the fate of the later formed disc. However, the egg cytoplasm does demonstrate strong influence on developmental parameters, such influences being termed "maternal effects", since the egg cytoplasm is substantially maternally derived. It will be useful to pause here to consider the development of the insect egg, or at least the *Drosophila* egg, the development of which is similar to that of most insects. Following fertilization, the nucleus divides eight times and the resulting nuclei migrate into the peripheral cytoplasm of the egg cortex or periplasm. Here they continue to divide to form an eventual syncitium with about 4000 nuclei. There now follows the production of plasma membrane, converting the syncitium into a multicellular mass with most of the distinct cells present in the surface layer. The embryo is now termed a blastoderm and normal gastrulation and development proceeds as in other organisms. With this form of development, strictly maternal effects can be expected up to the stage of 4000 cells or so. There is experimental evidence to suggest that maternally derived cytoplasm contains fractions which affect cleavage, gastrulation, germ cell commitment, sex determination, some of the exterior and interior orientation of larva and adult, and some primary organogenesis. These results are derived from experiments on maternal effect lethal mutants. These mutants have been extensively reviewed by King (1970). An example of such an effect is to be found with the inheritance of the gene rudimentary (r. 1–54·5). When hemizygous mutant males (male *Drosophila* have one X chromosome, females have two, so for genes carried on the X chromosome the males are said to be hemizygous) are crossed with homozygous mutant females, all the progeny die as embryos. If these hemizygous males are crossed with heterozygous females, however, all the progeny survive. The conclusion from such a mating pattern is that the heterozygous female provides the progeny with some cytoplasmic factor necessary for their survival.

Now, of the many genes known which betray a maternal effect, one group are of particular interest here and that is those which affect the form of parts of the insect cuticle via the imaginal discs. Mutants which cause alterations in the positioning of the integumental areas or lead to the development of the wrong piece of integument in the wrong place, i.e. an eye in place of an antenna or a thorax in place of a head, are termed homeotic mutants. Some homeotic mutants show a maternal effect. For example, the gene *bicaudal* (Bull, 1966) in *Drosophila* shows such a mating pattern. The affected progeny die as embryos because of head abnormalities and, in some cases, the head is replaced by another abdomen in mirror image symmetry to the first abdomen. It seems likely that, if the larvae survived to adulthood, the adults would also present as homeotic mutants. Another mutant, *tumorous* *head*, displays a clearer example of a maternally affected homeotic mutant. In affected flies, antennae are replaced by legs and parts of the proboscis may be replaced by genitalia (Postlethwait *et al.*, 1972). Actually, most larvae of affected stock die, but the surviving adults show these maternal effects which must, beyond doubt, operate through the imaginal discs. Such examples permit us to conclude that the determination of adult integumental structures via the imaginal discs may be affected by cytoplasmic factors conferred on the egg by the mother. Put in another way, there are non-chromosomal factors in the egg cytoplasm which may powerfully influence the determination of imaginal discs and adult integument and the pattern of gene expression which they display.

Complementing these experiments on maternal effects via the cytoplasm are the results of nuclear transplant experiments. Such experiments with nuclei from the syncitial blastoderm stage or even early gastrulae transplanted into unfertilized eggs (Illmensee, 1973) demonstrate that such nuclei are fully totipotent and will support normal growth and development of the resulting embryo at least through larval life and, in one case, to the pupal stage. Conversely, although the embryonic nuclei are totipotent, the embryonic cells are not. Mixing of genetically marked cells from anterior and posterior halves of blastoderm embryos with whole embryos, followed by culture and metamorphosis, leads to malformed animals (Chan and Gehring, 1971). The anterior embryonic cells from embryos at the blastoderm stage, when mixed with whole embryos and allowed to develop, produce only anterior adult cells.

This evidence from transplanted cells and nuclei now reinforces our earlier conclusion about maternal inheritance—namely that there are cytoplasmic components which are involved in maintaining or determining commitment to a particular developmental fate and that the nuclei themselves, if transplanted into a different cytoplasmic environment, can be freed from such a restriction. The genes which are affected by these very early factors are, however, rather few, although their effects may be frequently pleiotropic; other mutants are known which, through becoming effective at a later developmental stage, do affect the determination of the imaginal discs very profoundly.

2. Homeotic mutants

As we remarked in the previous section, homeotic mutants are ones in which one body part is replaced by another. The examples discussed earlier betrayed a maternal effect in their expression but now we will examine the main bulk of homeotic mutants which, though not maternally influenced, do affect the form of the adult via the imaginal discs. A table of such mutants is given by Postlethwait and Schneiderman (1972) and many are very dramatic. An interesting aspect of these mutants is that many of the genes involved appear to be clustered on the third chromosome of *Drosophila*. We will return to discuss that interesting observation later. To return to the main question of homeotic mutants, we ask first at what stage in imaginal disc formation the determination to become eye, antennae or whatever is fixed. A rather neat experimental trick can be used to mark cells at particular developmental stages in *Drosophila*, that is to induce a somatic crossover by X-rays. Such crossovers provide a permanent chromosomal genetic marker and clones of marked cells may be detected. Using such a technique, it has been found that in the homeotic mutant *Antennapedia*, in which the antenna is made up of a mixture of antennal cells and leg cells, clones of mixed cells may be detected when the marker is applied up to the third instar larva. In other words, up to that time in development, the commitment to antenna or leg is still relatively plastic and the homeotic mutant is still able to disturb the initial determination up to that stage.

Another very interesting series of homeotic mutants is described by Markert and Unsprung (1971), all affecting the organization of the adult thorax in *Drosophila*. Five different mutations are involved, styled a, b, c, d, and e. Mutation a causes the anterior portion of the adult metathorax (which normally bears the halteres) to resemble the anterior portion of the mesothorax (which normally bears the wings). And a mutant, therefore, bears recognizable but somewhat abnormal wings on the metathorax, in addition to the normal pair on the mesothorax. Mutation b causes the posterior portion of the mesothorax to resemble the posterior portion of the metathorax, i.e. to show some haltere development. Mutation c is often lethal, but such flies as do survive show a spectrum of homeotic changes similar to the range found in the other mutants. Mutation d causes the first abdominal segment to resemble a normal metathorax segment bearing halteres and legs. These flies, therefore, have four pairs of legs. The e mutants have the posterior portion of the metathorax similar to the posterior portion of the mesothorax, i.e. with somewhat aberrant wings as well as halteres on the metathorax. A number of very interesting observations with these thoracic mutants have been made in *Drosophila.* The first is that, as we have already remarked, the genes are all tightly clustered—crossing over between them is extremely rare. Moreover, the genes are situated so closely together that they affect one another in a form of *position effect.* This phemonenom can be demonstrated in one way by observing the effect of two of the mutants together in a cis/trans experiment, that is with both genes on one chromosone and the two wild type alleles on the other (cis), or one mutant gene and one wild type allele on each chromosome (trans). (Such closely clustered groups of genes have come to be called complex loci or pseudoalleles because of the wild type phenotype of the cis-position flies.) Of even greater interest is the finding that the position effect is polarized, each locus tending to affect the locus to the right and not to the left. Thus, when the mutant alleles are in the trans position (in the cis position they will normally be wild type) a combination of a and b genes will give a b phenotype, a b and c combination a c phenotype and so on. Presumably, the explanation for this phemonenon is that the wild type allele to the right of a locus is inactivated, and so the single dose mutant allele is expressed as if it were homozygous.

This type of position effect differs from a quite different genetic situation producing the mottled phenotype of white eyes in *Drosophila,* but confusingly also called position effect. The latter effect is apparently due to the influence of a mass of heterochromation on genes near to it. Homeotic mutations of the thorax series clearly involve alterations to the determination of particular imaginal discs. They also show that some eukaryotic gene arrangements lead to gene transcription being influenced by neighbouring genes, but in a unidirectional manner. If we try to ascertain exactly how the bithorax and other genes operate in affecting imaginal disc expression, it is hard to escape the conclusion that all the imaginal discs involved are relatively totipotent until the stage at which these genes become active, and that the function of the products of these homeotic genes is to suppress or constrict the potential developmental directives of the disc. In other words, abdominal segments do, at an early stage, have the potential to become thoracic segments and metathorax to be merothorax, in addition to their normal morphological destiny. If the suppressing genes are themselves inactivated or their influence diluted, then some of the original totipotency of the disc and the adult segment is realized. At the moment, it is not certain that this is the correct or only explanation for the bithorax series of homeotic mutants, but it remains a probable one. It certainly is a most important view of how gene expression of gross morphology may be controlled, since, in such a scheme, differentiation might operate by restricting gene expression, i.e. by suppressing all the "tissue-type master genes" bar one, and so permitting a cell and its progeny to turn on only one of the many programmes at its disposal.

3. Transdetermination

So far in this chapter the imaginal disc expression has been talked about as if it was always faultless, i.e. that any one disc or disc piece, even after prolonged culture in successive larvae or adults (both of which support imaginal disc cell proliferation without differentiation) would, when permitted to differentiate in a metamorphosing pupa, always reliably express its expected fate. Such fidelity of commitment is not absolute, however, and the exceptions, in which disc tissue is induced to express something other than its normal fate, is termed transdetermination (Hadorn, 1967) (see Fig. 19). It should be emphasized that transdetermination is a very rare event. Anal-plate clones have been maintained through 70 transfers, over a period of several years (Remensberger, 1968) and yet have differentiated faithfully into only anal-plate at the end of the day.

Certain characteristics of transdetermination are worth noting. First, any one imaginal disc can be induced, on rare occasions, to convert to almost any other type of disc in terms of its expression, but not in a haphazard way. Instead, there is a polarity, so that anal plate can convert to palpus more easily than to wing, but may convert to wing if sufficient cell division is permitted. It may even go through to a thoracic type disc expression, but that takes even longer. Once a disc has been transdetermined, it may revert, but more frequently the new committant becomes fixed or transdetermination in a further forward direction transpires. This aspect has been discussed by Gehring (1968). What is the mechanism underlying transdetermination?

Sonatic mutation has often been advanced as a likely explanation and is certainly difficult to exclude. However, the changes are rather too frequent to be easily squared with mutation and they also appear to be directional rather than random. In addition, transdetermination is often a multicellular response, so that a disc

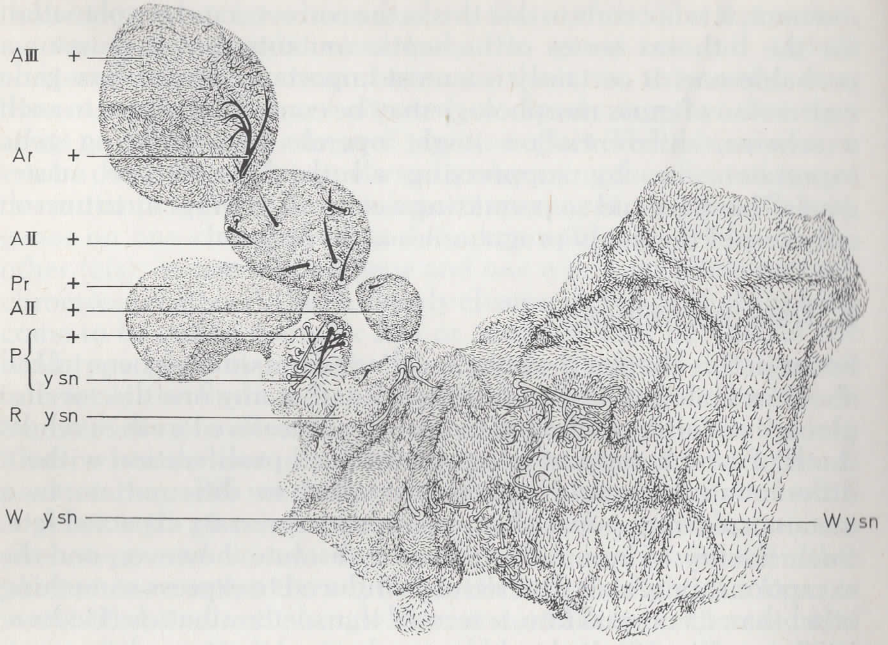


FIG. 19. Transdetermination in a clone of marked cells derived from a culture of antennal imaginal disc from *Drosophila*. The clone includes part of the palpas (Py SL) and rostral membrane (Ry SL) which have given rise to the allotypic wing (Wy SL) by transdetermination. The remaining parts which remain unaltered include A11 — the second antennal segment, AIII the third antennal segment and Pr-prefrons. Parts designated with (+) show wild type colouration and parts designated YSL are colour marked (from Gehring, 1972).

with a new fate is found to derive from more than one original disc cell (Gehring, 1967). Although somatic mutation does not exactly fit the facts, however, no other mechanism has, as yet, proved to be causal. Whatever process is involved, it seems to be largely dependent on cellular proliferation. The more the disc grows in cell number, the greater the likelihood of detecting transdetermination.

Although transdetermination cannot be adequately explained, it should be noticed that it has some characteristics in common with the homeotic mutants previously discussed. In particular, the expression of the mutants and the transdetermined discs both show a polarized series. This surely lends weight to the argument

already advanced that the expression of genetic determination is a matter of reducing potential by the production of repressing molecules and that a cascade of tissue commitments may exist, so that suppression of one tissue-type master gene automatically alters the commitment and brings the cell under the influence of a different master gene.

4. Phenocopies

A phenocopy in genetical terms is a mimic of a mutant phenotype, produced by treatment of a wild type with some deleterious agent at a sensitive stage in its development. Many of the agents used are likely to be active on the genetic material itself, e.g. X rays, fluorouracil and nitrogen mustard. Treatment of *Drosophila* 3rd instar larvae with these agents produces a proportion of adults in which parts of the antenna are replaced by leg. Phenocopies are often employed in genetical studies as providing a key to understanding the *modus operandi* of the mutant gene which is copied. In particular, it is argued that the period in which the developing insect is sensitive to these agents may well coincide with the time at which the structures affected are determined. Unfortunately, different stocks of wild type *Drosophila* respond to the application of these phenocopy agents in somewhat different ways, some developing one abnormality repeatably while another stock persistently displays a different abnormality (Goldschmidt and Piternick, 1957). Nevertheless, the existence of phenocopies to many *Drosophila* mutants, in particular to homeotic mutants involving imaginal disc determination, does prove that external influences affect imaginal disc determination during development. The agents may well act by affecting cell death, cell division or the rate of cell proliferation (somatic mutation can be discounted as the mode of action of phenocopy agents), thus emphasizing the important role of cell division in controlling the expression of the genes involved in tissue determination.

5. Specification

Insect imaginal discs offer another advantage to the experimental biologist besides the long time lag between determination and differentiation. This is that different parts of a disc, when dissected away and permitted to differentiate independently in a new post, develop into distinct parts of the piece of integumental jigsaw puzzle. Clearly different cells within a disc differ from one another, depending on where they are located. Since the adult integument is liberally adorned with bristles and hairs, the different parts of a disc area may be mapped out into a spatial field and the fate of each cell within a disc analysed. A considerable literature has built up around this subject (see review by Postlethwaite and Schneiderman, 1973; Bryant, 1973) but only the main findings need be recounted here. They are, first, that, unlike the determination of the fate of the whole disc, e.g. to be mesothorax or metathorax, the commitment to development into a particular bit of the spatial field is not necessarily passed on by cell division. If disc cells are allowed to proliferate in a larval or adult test, the progeny of a single cell may develop into different parts of the disc field; i.e. different from the part which was the destiny of the original cell. Bryant (1973) has proposed the term specification for such a localized commitment in order to distinguish it from the stronger determination of the fate of the whole disc which is, indeed, passed on faithfully by cell division within the disc. Persistence of specification after limited labile division is well illustrated by the recent work of Garcia–Bellido *et al.* (1973). The second observation is that the manner of providing the pattern of the field is probably similar in all discs, since mutants with similar disturbances of the field pattern have been discovered for many different discs (Wolpert, 1969, 1972). An interesting conclusion can be drawn from these data which, although only of secondary relevance to our topic, is worth mentioning here. It is that the positional information possessed by any cell of the disc is probably provided for the cells by chemical gradients within the disc. Such stimulus would enable individual cells to know where they were in the disc and, therefore, into which part of the final pattern they would finally develop. Such a conclusion is enhanced by the finding that individual cells of a disc, if disaggregated, will, on reaggregation, tend to recover their original places (Garcia–Bellido, 1972). It may be that positional information and pattern formation are not primarily genetic phenomena, but may be mediated by cell surface recognition between cells and molecules. But to the extent that the information affects the expression of the genes, it must come to be translated into genetical terms. Specification in imaginal disc cells is, therefore, an excellent example of what must often be the final and most flexible part of the control of differentiation, the response of a determined cell to the environment influences of its neighbouring cells and molecules.

6. Comment

Studies on imaginal discs and analysis of the many mutants associated with them provide perhaps the best opportunities for studying determination, i.e. the commitment of a cell to a destiny before the gene products involved in that destiny are in production. The other aspect of differentiation styled specification is also an attractive part of insect imaginal disc biology.

The view of the genetic control of differentiation which imaginal disc studies provide is most important. It is that a differentiated cell does, for the most part, become determined or fixed to its fate early in development and that the state of determination is dynamic, involving a stable equilibrium reached between cytoplasm and nucleus which commits the cell to follow a particular pathway. This equilibrium is not easily broken, but if it is, say by prolonged cell division or by particular extrinsic factors, then the cell may be shifted down into another state of stable equilibrium appropriate to a different developmental pathway. The phrase "shifted down" is intended to indicate that different differentiated fates are related and a cell may move from one to another only under severe constraints. Such a model not only accounts for the comparative totipotency of nuclei removed from determined cells and implanted into a new cytoplasmic mileu, but also predicts the existence of cytoplasmic repressors and master genes for particular differentiated fates. These ideas will be discussed at greater length in Chapter 7.

G. Higher plants

Comparatively few experimental systems have been developed for the study of gene expression which utilize material from higher plants. Of the ones which are utilized, many are primarily concerned with the mode of action of plant hormone, which is discussed in Section 7C. The lack of clearly compartmentalized organ differentiation in plants means that there are few opportunities for isolating one kind of differentiated plant cell in bulk. Moreover, the cellulose cell wall of plants renders the isolation of cellular fractions more laborious than in most animal tissues. However, certain phenomena which have a bearing on the control of gene expression are probably best exemplified in plant systems and these will be briefly discussed.

1. Growth of plants from isolated somatic cells

The establishment of plant tissue cultures derived from single cells was first accomplished by Muir *et al.* (1958). They successfully isolated single cells from growing cultures of tobacco plant callus tissue and grew on clones of cells from these single cells. Although Muir actually grew his single cells on top of pieces of callus tissue, subsequent work has proved that isolated tobacco plant cells will grow and divide in culture to produce clones of plant cells (Jones *et al.*, 1960). The most famous plant tissue culture experiments are, no doubt, those of Steward, in which complete carrot plants were reared from isolated phloem parenchyma cells (Steward *et al.*, 1964; Steward and Krikorian, 1971). Although the precise state of differentiation of the isolated original cell is uncertain, these and other similar experiments with tobacco plant tissue provide an impressive example of the genetic and developmental totipotency of a tissue cell. No equivalent example exists for an animal cell, although nuclei from differentiated cells will sustain normal embryonic growth and development, if implanted in enucleated eggs (see Section 4E). Whatever the restriction of gene expression involved in the phloem cell concerned, it is capable of reversal and alteration so that the full genetic potential of the plant may be realized.

2. Crown gall in plants

There are a number of plant diseases which resemble, at least in some ways, the tumours of animals. The best known is crown gall. This disease affects many different species of dicotyledonous plants and is initiated by a tumour inducing principle (TIP) which is elaborated by a specific bacterium, *Agrobacterium tumefaciens.* TIP accomplishes the cellular transformation to the crown gall conditions very rapidly, i.e. within about three days, and following transformation, the crown gall cells will continue in abnormal autonomous proliferation quite independently of the bacterium and may be maintained in culture indefinitely. The topic has been reviewed by Braun (1962, 1965, 1970).

Similarities between plant diseases such as crown gall and animal tumours have often been pointed out (Braun, 1965). In particular, tissue cultures of the crown gall cells will grow profusely on relatively simple media which do not support growth of the normal healthy plant tissues and implantation of the cultured crown gall cells into a healthy plant results in development of a new crown gall growth. There are, however, a number of important parameters on which crown gall plant cells and animal tumour cells differ. The first is that of invasive growth. Admittedly, it is difficult to assay for invasiveness in the non motile cellular anatomy of a growing plant, but it is certainly an important criterion in determining the neoplastic character of animal cells. Another important difference is the speed of induction of crown gall in the presence of the inducing bacteria and the TIP which they release. No true tumours of animals show similar pathways of induction. The mechanics of crown gall induction are themselves interesting, since prior to complete induction, the plant affected by the attacking bacteria must be conditioned by wounding and if cells are isolated from the healing wound, their tendency to grow as crown gall cells is gradual, becoming most pronounced only after three days. Moreover, the degree of transformation is transmissible, since partially transformed crown gall cells in culture give rise to similar cells (Braun, 1965).

Crown gall cells are clearly caught in an unusual differentiated state and, in this state, are remarkably stable. But then, not all stable but relatively undifferentiated cell states are tumours. If crown gall cells are compared with the cells of insect imaginal discs, some parallels can be detected. Is it then possible to find circumstances in which fully transformed crown gall cells will differentiate into normal plant tissues? So far, such circumstances have not been found (discussion of a related problem in teratoma tumours of mammals will be found in Section 5A). However, if partially transformed plant cells are used, a different prognosis is valid. Such partially transformed cells are those which result from brief exposure of dividing plant cells to TIP and, instead of displaying the characteristic unorganized crown gall growth, such cells produce plant teratomas, somewhat chaotic mixtures of partly differentiated tissues and organs. Such cells will grow indefinitely in tissue culture, still tending to form partly organized plant structures. When transplanted into a growing plant, they will also produce clusters of abnormal leaves or buds. If further transplants are made from such buds onto other healthy plants, more normal growth results, and serial transplantation will eventually dilute out any tendency towards unorganized growth (Braun and Meins, 1970). It can, therefore, be concluded that the partly transformed cell can be induced to differentiate into a normal plant cell.

A strikingly similar picture emerges from experiments with a transplantable tumour which is invariably produced by some hybrid tobacco plants, resulting from a cross between *Nicotiana glauca* and *Nicotiana langsdorffi* (White, 1939). These abnormal cells closely resemble crown gall cells in that they will grow profusely on simple media and are readily transplantable. However, as with the partially transformed crown gall cells, these tobacco tumour cells can be switched back to organized growth by altering the culture conditions or by transplantation into a new host plant. There is also good evidence that this conversion is largely dependent on plant hormone mediation (discussed by Braun, 1965). Whether these abnormal types of cells in plants are directly comparable to animal tumours is arguable, but certain deductions can be drawn from them which relate to gene expression. The most important is that many cellular states are extremely stable and that apparently poorly differentiated cells may prove as much in equilibrium as many differentiated cells obviously are. Whether by self control involving hormone synthesis, or by other means, particular genetic programmes have high capacities for self-maintained equilibrium.

3. Phase changes in ivy (*Hedera helix*)

As pointed out by Brink (1962) and Wareing (1971), many woody plants undergo dramatic changes of phase, in that, for the first few years of life, the plant displays juvenile characteristics and does not flower, but, at anything from 1–30 years, the plant is rather suddenly transformed to an adult shrub or tree, develops adult leaves and is able to flower and fruit. The two authors mentioned here both pointed out similarities between this process and the phenomenon of vernalization.

An interesting example of this phase change in a woody plant, involving a transition from a juvenile to an adult form, is provided by the common ivy, *Hedera helix* (see discussion in Wareing, 1971). If ivy is grown from seed the young plants have lobed, palmate leaves, climb vigorously, have hairy stems, possess anthocyanin pigment and do not flower. After some years, and particularly when the growth of the plant has reached eight feet or so, the transition to adult form occurs and, especially in the upper part of the plant, the leaves are entire and ovate, the stems smooth, the plant ceases to display a climbing form in its new growth, anthocyanin is absent and flowers and fruits are produced. The final and revealing observation is that, if cuttings are taken from the adult plant, they continue to display the adult character, producing new dwarf plants with little tendency to climb, ovate leaves and a ready capacity for flowering. Interestingly, this phase change was well known to Victorian gardeners, who used dwarf plants of mature ivy to provide flowering bushes along the sides of driveways.

Studies on the phase change in ivy have revealed that callous tissue derived from juvenile and adult ivy behaves differently in tissue culture, the juvenile tissue having a high growth rate and a marked tendency to form roots, while the adult tissue grows more slowly and shows little root regeneration (Stoutemeyer and Britt, 1965). It has also proved possible to accomplish reversion of adult phase to juvenile phase by treatment with gibberellic acid or exposure to elevated temperatures (Stoutemeyer and Britt, 1961).

These phase changes within a single plant are obviously not examples of permanent alteration in the genome, since conversion from one state to the other is possible, but they are situations of extraordinarily stable states of restricted genetic expression. Robinson and Wareing (Wareing, 1971) have experimented with tissue derived from intermediate transitional parts of ivy plants. Such plants were juvenile at the bottom and adult at the top. Cuttings from the transitional zone displayed a mixture of characters and, although some reverted to a truly adult or juvenile phenotype with time, others retained their intermediate character. Such stable intermediates certainly argue against a simple and single genetic switch being responsible for the developmental transition.

Ivy is by no means exceptional in displaying marked phase changes during development and very many woody trees, particularly conifers, display changes in branching and leaf distribution with age and growth, these changes being retained by cuttings taken from the appropriate part of the plant.

It seems that stable differentiated states are common in plants and are often difficult to distinguish from changes in genetic structure, e.g. the witches broom, which are common aberrant growths on birch and other trees. Very stable patterns of gene expression will tend to be difficult to distinguish from changes in gene structure or composition, except by careful breeding experiments. It would be easy to visualize classes of gene control mechanism which might be stable throughout meiosis and would, therefore, prove indistinguishable from true mutations. The next topic in this section illustrates this difficulty very well.

4. Unusual genetic elements in maize and *Antirrhinum*

Dr. Barbara McClintock has worked for many years on some very unusual genetic phenomena in maize (*Zea;* McClintock, 1968). Certain genes, affecting the colour of the kernel (see Fig. 20) or of the plant itself, appeared to mutate with very high frequency in some maize strains. This was traced to a position effect, the expression of the particular genes being affected by a genetic element which was inserted in the genome near, but not necessarily adjacent to, such genes. Moreover, the genetic element causing the effect proved to be highly mobile, being able to appear at new locations in the genome (with equivalent position effects on neighbouring genes) and disappear from previously determined locations. The genetic unit causing the position effect was termed Dissociation (Ds) by McClintock and proved to be affected by, and indeed activated through, an unlinked dominant gene termed Activator (Ac). Both Ds and Ac proved capable of frequent and dramatic changes in locus in the genome.

Another very curious fact about the Dissociation gene is that when its location is altered, the position effect in the previous locus is lost and previously neighbouring genes return to their original phenotypic expression. That in itself is surprising, but even more remarkable is the fact that the effects of Ds on neighbouring genes is to produce phenotypes which are identical with known mutant alleles of these genes (McClintock, 1957). This seems to pose the question of whether all mutations are what they are taken to be—irreversible alterations in DNA base sequence. Actually, it is not improbable that these genes affect other loci by heterochromatin position effect, as is known to occur with certain loci in *Drosophila,* and certainly Ds is known to be associated quite often with chromosome breakage or other structural changes at its locus.

McClintock has termed these genetic elements "controlling elements" and, certainly, there is no evidence that they code for proteins. Their relation to chromosome breakage and their property of changing location within the genome suggests some parallels with integrated viruses, or other episomic elements found in the bacterial genome.

Although maize has proved the most productive system for studies of such unusual controlling elements in the genome,

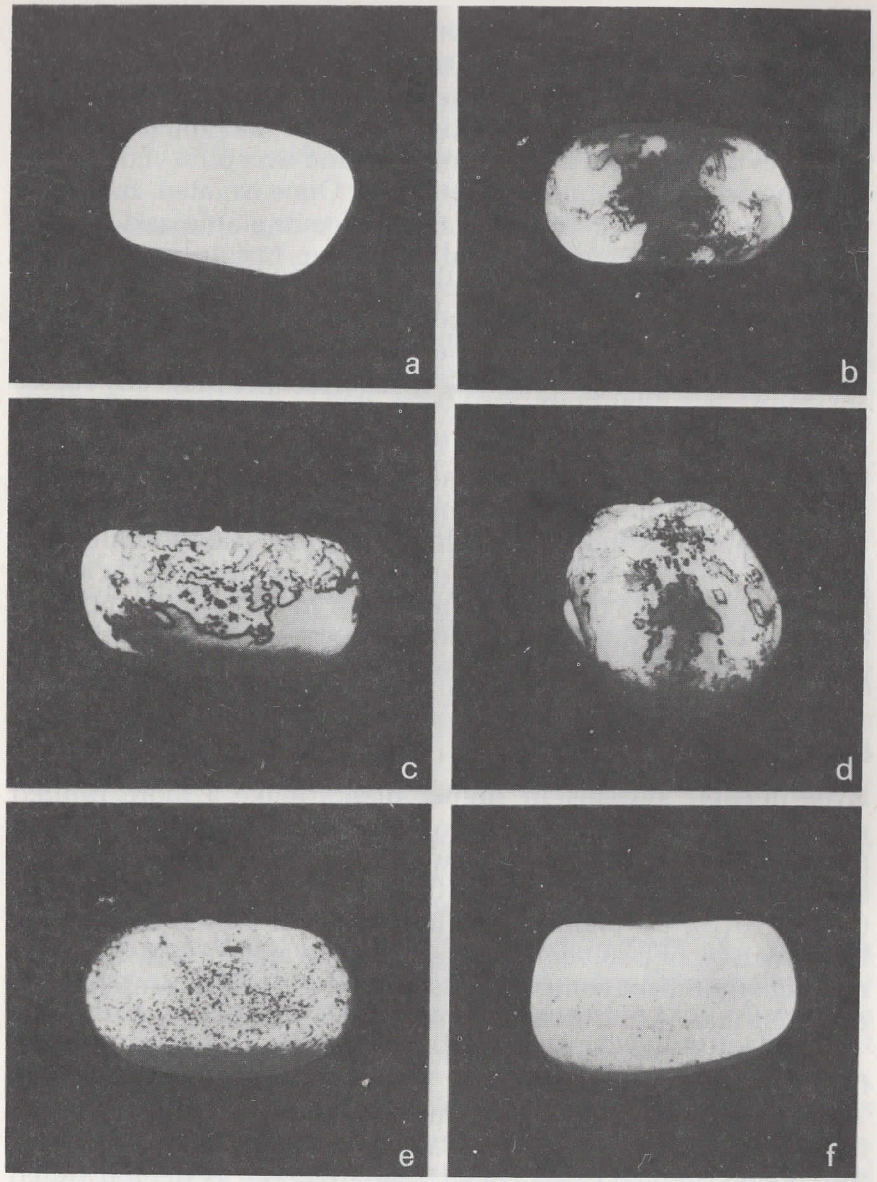


FIG. 20. Photographs of kernels of maize *Zea,* showing the various types of colour patterns produced by chromosome breaks involving the elements "Ds, or Dissociation" and "Ac, or Activator". Fig. (a) lacks Ac, and Ds is not effective. (b) (c) and (d) are various forms of Ds activity in the presence of one Ac gene, Fig. (e) shows Ds activity with two Ac genes present and (f) with three Ac genes present (reproduced with permission from McClintock, 1957).

rather similar phenomena have been reported in other plant systems and particularly in *Antirrhinum* by Fincham (1970). In studies on colour breaks in the pigment distribution of *Antirrhinum* flowers, Fincham and his colleagues find apparant mutation rates of several % in the germ cells and over 50% of the tissue cells may display a mutant phenotype. Once isolated, the mutations appear to be genuine in that they are stable and display regular mendelian segregational behaviour. Not only is evidence about controlling elements in eukaryotic genomes of great interest in itself, but the curious copying of true mutations in the phenotypic expression of these elements, together with their comparative stability over many generations, renders any future evidence of very great interest. Normal mutations may not be always what they have been taken to be, e.g. the similarity between homeotic mutants and transdetermination in the developmental biology of *Drosophila* imaginal discs (see Section 5F), is another situation in which true and pseudo mutations present similar phenotypes. The apparent requirement for a high but controlled rate of directed mutation in explaining antibody diversity (see Section 3A) is another situation where a controlling genetic element would prove useful.

5. Comment

Experimental systems in higher plants make a more limited contribution to the knowledge of gene expression than do those on higher animals. Some of the work on plant hormones and their action is discussed in Section 7C; work on plant ribosomal RNA (Ingle, 1973), on plant cell cultures (Street, 1973) and on the biochemistry of plant embryology (Steeves and Sussex, 1972) are making significant contributions, but have been less intensively explored than the equivalent systems in animals.

The plant systems, discussed in this section, do make important contributions to the view of eukaryotic gene expression. They underline, on the one hand, the stability of the differentiated state, as in the ivy experiments, and on the other, the totipotency retained by the differentiated cell, as demonstrated by the crown gall and carrot cell work. Differentiation is clearly a state of extremely stable equilibrium between nucleus and cytoplasm, but it is certainly possible to break the stability in favour of development in a new direction.

The controlling genetic elements discussed in research on maize might well explain phenomena in other biological situations. In particular, the apparently high incidences of radically altered gene expressions found in transdetermination of *Drosophila* imaginal discs and in the development of clones of antibody forming cells synthesizing novel immunoglobulin might involve the operation of such elements.

H. Mitochondria and chloroplasts

Almost all eukaryotic cells possess mitochondria and many contain both mitochondria and chloroplasts. These organelles, perhaps the remnants of symbiotic prokaryotic cells within the eukaryotic cell, possess their own genome and make many of their own proteins. They, therefore, represent a distinct and probably differentiating genetic system within the eukaryotic genetic system, enjoying some independence but also being involved in some measure of co-operation. Gene expression of the mitochondrial or chloroplast genome and its relationship to the genetic expression of the whole cell has been analysed in only a few cell types. The most productive experimental systems, each of which we will discuss separately, are the mitochondrial mutants of budding yeast, normally termed the petite mutants, and the chloroplast mutants of the unicellular alga *Chlamydomonas.* Both systems are particularly useful because they offer the possibility of analysing the inheritance of cell lines with the same nuclear genes but differing mitochondria or chloroplasts.

Before discussing these experimental systems, however, the present state of knowledge of the genetics and gene expression of mitochondria and chloroplasts will be summarized.

1. Mitochondria and chloroplasts contain DNA of a different base composition to the nuclear DNA, permitting isolation of distinct satellite DNA bands by centrifugation through a caesium chloride density gradient. Animal cell mitochondrial DNA is about 5 μ long—some 15 000 base pairs—and circular, but in higher plants, yeasts, *Neurospora* and *Tetrahymena,* it is larger, being up to 25 μ long and in some of these cases probably not circular. Chloroplast DNA is between 50 and 150 μ long, frequently linear, but sometimes circular (Tewari and Wildman, 1970).
2. Mt (mitochondrial) and Chl. (chloroplast) DNA replicates out of phase with the main S period of the host cell cycle.
3. Mt and Chl. DNA is, like prokaryotic DNA, apparently unconjugated to basic protein.

4. Ribosomes of mitochondria and chloroplasts are distinct from those of the host cell and the ribosomal RNA genes for these organelles are present on their own genome.
5. Some mitochondrial transfer RNA molecules are distinct from those in the rest of the cell and some are coded on the mitochondrial genome. N-formylmethionyl transfer RNA occurs only in bacteria and eukaryotic mitochondria.
6. About 80% of mitochondrial protein and a lesser percentage of chloroplast protein is synthesized on cytoplasmic ribosomes (including the cytochrome enzymes), the remainder on the mitochondrial ribosomes (Ellis and Hartley, 1971).
7. The morphology of mitochondria and chloroplasts is variable, both between tissues of the same organism and between, say, an amoeba and a mammalian cell (for the mitochondria, that is). Put in other terms, mitochondria and chloroplasts undergo some morphological differentiation even in differing tissues of one organism. Whether this differentiation is accompanied by differential expression of the mitochondrial or chloroplast genes is not known, but seems probable.
8. The nuclear genome of *Xenopus* (and probably many other eukaryotic organisms also) does not possess a copy of the mitochondrial DNA (Dawid and Blackler, 1972).
9. Both mitochondria and chloroplasts are often exclusively maternally derived, either by failure of the sperm mitochondria (or mitochondrion) to penetrate the egg at fertilization, or because of its preferential breakdown following fertilization. Mammalian mitochondrial DNA is no exception to the rule of maternal inheritance (Hutchinson *et al.*, 1974).
10. A number of mitochondrial mutants are known, each of which displays some sort of maternal inheritance. For example, clones of erythromycin resistant *Paramecia* have been reared by Beale (1969) and these cells donate their resistance to their partner cells at conjugation.
11. Mitochondria and chloroplasts probably possess distinct polymerase enzymes. A unique RNA polymerase has been isolated from the mitochondria of *Neurospora* (Kuntzel and Schafer, 1971).
12. Both chloroplasts and mitochondria are probably invariably derived from similar pre-existing organelles (Gault and Arnott, 1963; Luck, 1963).
13. The number of mitochondria and chloroplasts within a eukaryotic cell is probably as variable as between different

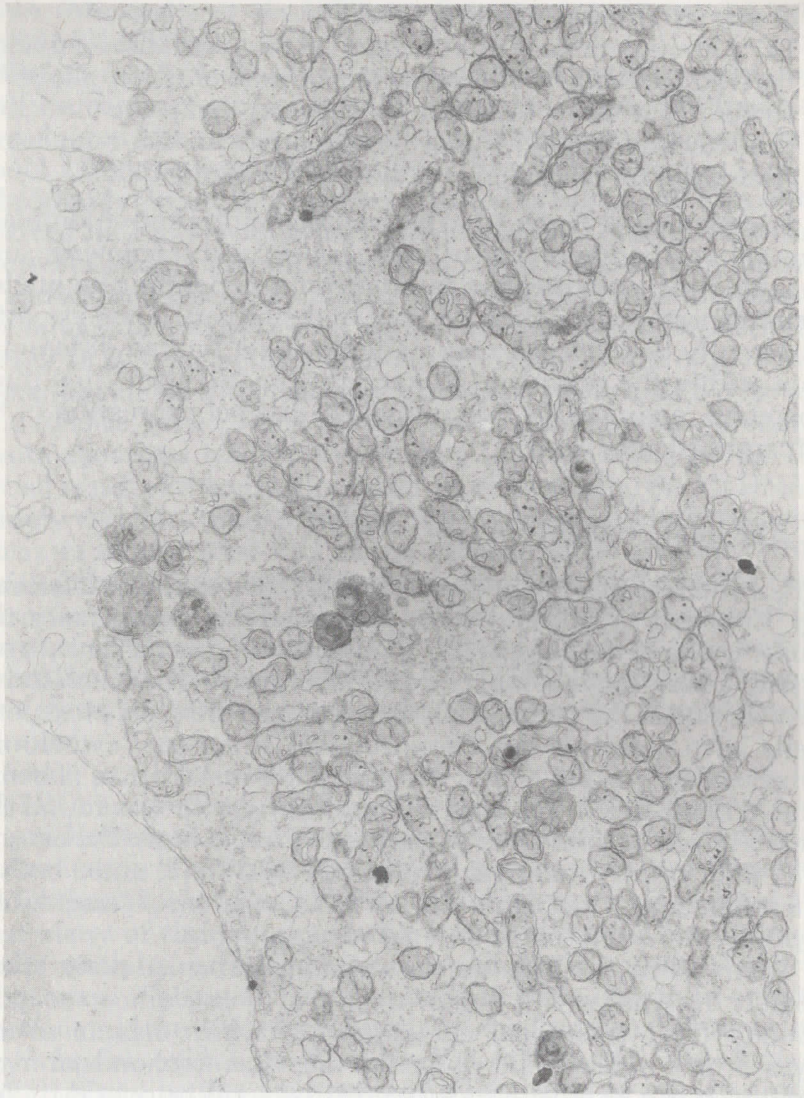


FIG. 21. Electron micrograph showing part of a *Xenopus* oocyte. Part of the nuclear envelope appears in the lower left-hand corner. The cytoplasm, occupying most of the picture, is filled with a dense aggregation of mitochondria. This is part of the characteristic "mitochondrial cloud" which appears near to the nucleus in these cells (photograph kindly provided by Dr. F. Billett and Mrs. E. Adam).

tissues and species (see Fig. 21). In some cases, it may be as low as one (Hoffmann and Avers, 1973).

14. Although there is no evidence yet for translation of nuclear derived messenger RNA on mitochondrial or chloroplast ribosomes, there is evidence which suggests that some mitochondrial messenger RNA may be exported to the cytoplasmic ribosomes for translation (Attardi and Attardi, 1968).

In summary, it is clear that both mitochondria and chloroplasts are extremely important cellular constituents, enjoying some genetic independence, but in other ways depending on nuclear gene expression for their welfare and survival. They are often maternally derived and may undergo some limited differentiation within the organism. Eukaryotic genetic expression must, therefore, embrace the important and distinct expression of the Mt and Chl. genome.

1. The petite mutant in yeast

These interesting mutant strains of yeast cells were first described by Ephrussi (1953) and their non-mendelian inheritance discussed. The petite yeast cell is commonly of normal size but gives rise to a very small colony, no doubt because of the much slower growth rate of the petite as compared to the wild type. Most petite cells have defective mitochondria, lack functional cytochrome enzymes and respire solely by glycolytic metabolism (Thomas and Wilkie, 1968). Soon after their discovery, it was found that yeasts could be converted to the petite form at high efficiency by u.v. light or acridine dyes (Ephrussi, 1953) and, since both of these mutagens act directly on DNA, the involvement of a cytoplasmic nucleic acid seemed very probable.

Neurospora also boasts a class of mutant, named poky, which closely resembles the petite mutant of yeast, since the poky mutant lacks functional cytochrome enzymes, has an altered cytochrome oxidase and possesses altered mitochondrial membranes. It can also be induced readily by acridine dyes (Mitchell and Mitchell, 1952; Woodward and Munkres, 1966).

Since mitochondria are passed on in petite yeasts as a cytoplasmic factor and, as far as is known, mitochondrial conjugation does not occur, intensive recombination analysis of mitochondrial genes is not possible. If rare cytoplasmic heterozygotes arise mitochondrial genes of yeast, they are presumably rapidly dispersed and eliminated by somatic segregation. Actually, some limited recombination has been observed with mitochondrial DNA (Michaelis *et al.*, 1973), so future progress may be more rapid in this field.

Only a few particularly relevant aspects of petite yeast biology will be discussed here. First, the high mutation rate for these genes should be stressed. There is a spontaneous mutation rate of about 1% to the petite form and, by the use of mutagens such as ethidium bromide, this has been pushed as high as 99·8% petites derived in 3·2 cell doublings (Sloninski *et al.*, 1968). This is an incredibly high mutation rate and clearly deserves intensive investigation. Does it result from lack of DNA repair enzymes in the mitochondrial milieu, or some particular instability of the Mt DNA? In fact, the petite mutation can also result at a much lower mutation rate by a nuclear gene mutation, presumably by damage to a nuclear gene coding for a mitochondrial protein—probably a mitochondrial ribosomal protein (Goldring *et al.*, 1970). The actual result of the mutation in terms of damage to the Mt DNA is widely variable, often resulting in recognizable deletions, sometimes in almost total loss of the mitochondrial genome. When limited deletion occurs, the remainder of the Mt DNA has been observed to compensate for the loss by repetition of some of the remaining genes (Netter and Slonimski, 1973) as assessed by hybridization studies on the Mt DNA. This observation suggests that there is some measure of control over the size of the Mt genome.

Since yeast cells must often possess only one copy of the Mt DNA and indeed many possess only a single large mitochondrion (Hoffmann and Avers, 1973), earlier ideas about simultaneous mutation of many mitochondrial genomes are now obsolete.

Another aspect of petite genetics which deserves comment is that many of the proteins active within the mitochondrion are made partly on cellular ribosomes and partly on mitochondrial ribosomes. This statement means that some are multi-subunit proteins and the differing subunits are made in two different sites. Cytochrome oxidase is assembled in this way (Tzagaloff *et al.*, 1973). Many petites are apparently incapable of mitochondrial translation and loss of the Mt ribosomal RNA genes seems to be the commonest cause of the petite phenotype. The production of petites at relatively low mutation rates by a nuclear gene mutation is, probably, to be seen as a lesion affecting a protein of the mitochondrial ribosome (Goldring *et al.*, 1970). Other proteins which are inactive or absent from petites probably require processing in the mitochondrion to produce an active form, although actually made on the cytoplasmic ribosomes—aminolevulinic acid synthetase enzyme is an example of such an arrangement (see discussion in Maclean and Jurd, 1972).

A further and puzzling observation in petite yeast relates to the discovery of suppressive petite mutants (Ephrussi *et al.*, 1955). Such mutants can be induced to cross with wild type cells, forming ascospores. The spores of the petite diploid cells are not normally viable, but viable ascospores are formed by the diploid wild type cells following the petite/wild type cross. Such ascospores, on germination, give rise to colonies of cells which may be petite and in some crosses, all resulting colonies are petite. Such an observation implies not only a mendelian dominance for the petite mutation, but also an inability to express the wild type allele. It may be that the mitochondrial DNA of these petites is preferentially replicated in the cell, or that Mt DNA recombination somehow results in actual loss of the wild type copy of the relevant gene.

It might well be asked why mutations analogous to petites are not detected in higher organisms. Presumably, the reason is that they would normally be lethal and the non-lethality in the yeast cell is a direct result of the ability of these cells to grow permanently by glycolytic fermentation in culture. The absence of petites in isolates of wild yeast probably reflects the considerable selective disadvantage of the petite mutant in competition with the more vigorous growth of the wild type cells.

2. Chloroplast genetics in *Chlamydomonas*

Chlamydomonas is a unicellular motile alga living in fresh water. Different mating types exist which may fuse to form a zygote. A meiotic division then leads to the production of further haploid cells termed zoospores (which divide mitotically to produce a clone of new algal cells). Chloroplast genetics has been studied intensively by Dr. Ruth Sager in *Chlamydomonas reinhardi* (Sager, 1972). Many of the basic observations made on the petite yeast system have been paralleled by studies on *Chlamydomonas* chloroplasts. For example, the chloroplast ribosomal RNA is coded by Chl. DNA (Surzychi, 1969) and, although many of the respiratory chain enzymes of the chloroplast are synthesized on cytoplasmic ribosomes, one soluble protein, ribulose diphosphate carboxylase, is apparently made on chloroplast ribosomes (Ellis and Hartley, 1971). Whether this protein is also coded by a Chl. DNA gene is not proven, but seems probable. There is also good evidence that some of the ribosomal proteins of the 70S Chl. ribosomes are coded by Chl. genes and that mutants, displaying resistance to streptomycin, erythromycin and carbomycin, have a lesion in the Mt DNA (Sager and Lane, 1972).

Three aspects of the *Chlamydomonas* chloroplast experiments are of special significance and value. The first is that these algae demonstrate a purely maternal inheritance of chloroplasts, despite the fact that the zygote involves complete fusion of both male and female (+ and – mating strain) algal cells. This implies preferential destruction of the male chloroplast and its DNA. Sager and Ramanis (1967) have shown that the destruction of the male chloroplast DNA may be inhibited by irradiation of the female gamete just prior to fusion and that photoreactivation of active elimination occurs in visible and u.v. light. Sager (1972) suggests that the female gamete synthesizes a substance which emzymatically attacks the chloroplast genome of the male chloroplast. This mechanism is not unlike the modification/restriction system operating in some virus infected bacteria (Boyer, 1971). In these bacteria some cells act as restricting hosts and elaborate restriction eyzmes such as endonucleases are present which specifically cut unmodified DNA into short sequences. Other cells act as modifying host cells and make a methylase enzyme which methylates the DNA, so rendering it insensitive to the endonuclease. It is conceivable that the maternal Chl. DNA is temporarily methylated to prevent its destruction by endonucleases which destroy the Chl. DNA donated to the zygote by the male gamete.

The other aspect of *Chlamydomonas* genetics that is particularly interesting and relevant follows on from the maternal chloroplast transmission system. It is that, by inactivating the destructive factor with u.v. light, zygotes may be obtained which are heterozygous with respect to chloroplast genes. Such zygotes may occasionally be detected as occurring spontaneously, but suitable u.v. irradiation raises the yield of such heterozygotes to nearly 100% (Sager and Ramanis, 1967). Exploitation of this technique has enabled Sager and Ramanis (1970) to construct a genetic map of the chloroplast genome of *Chlamydomonas* with relative positions of eight genes. Mapping was made possible by the recombination of the Chl. DNA in zygotes heterozygous for the Mt DNA. Clearly, the technique can be extended as more mutants are isolated. By other techniques, the Chl. DNA of *Chlamydomonas* has been found to possess exclusively the chloroplast ribosomal RNA sequences, while chloroplast DNA polymerase, DNA-dependent RNA polymerase and some of the ribosomal proteins are synthesized and probably coded outside the chloroplast (Surzycki *et al.*, 1970). Many of the chloroplast transfer RNA genes are probably located in the Chl. RNA in this alga, as they have been shown to be in the tobacco plant (Tewari and Wildman, 1970).

A third observation on the genetics of the *Chlamydomonas* chloroplast deserves comment. It is that, following zygote formation, the chloroplast DNA peak displays a shift in density from 1·694 g/cm^3 to 1·689 g/cm^3 (Sager and Lane, 1972). This shift in density does not seem to result from addition of the male chloroplast genome and, indeed, there is good evidence that this is destroyed. The shift observed is resistant to pronase, α amylase and both pancreatic and T1 ribonuclease. Considering the remarkable adjustments detected in the mitochondrial genomes of petite yeast (Netter and Slonimski, 1973), this may represent a temporary gene amplification. Another possibility, discussed by Sager and Lane, is that it represents methylation of the DNA and, as discussed above, this might be a device designed to protect the female chloroplast genome while the enzymatic destruction of the male chloroplast genome was in progress. The selective advantage of destruction of the incoming chloroplast genome, ensuring exclusively maternal inheritance of the chloroplast, is not easy to understand. Clearly, recombination within the chloroplast DNA is thereby prevented and perhaps advantageously so.

3. Comment

Studies on gene expression in relation to chloroplasts and mitochondria are of very great value. Not only do they provide information about both transcriptional and translational control within the organelle, but also of co-operation between nuclear and cytoplasmic genomes and preferential affinities of chloroplast, mitochondrial and cellular ribosomes. The variation in the size of the genome of those organelles also promises that, when all the genes on the mitochondrial genome are mapped and answered for, it will provide a good basis for extended studies on chloroplast genomes. Certain questions seem to deserve close

study. They are:

1. Why is mitochondrial DNA so susceptible to mutation, as in petite yeast?
2. What are the mechanisms controlling gene replication and the replacement of lost portions of the genome in petite yeast?
3. Is differential gene expression an aspect of the differentiation of chloroplasts or mitochondria within one organism?

6. RNA Involvement in Gene Expression

A. Ribosomal RNA 250
B. Transfer RNA 257
C. Heterogeneous nuclear RNA 258
D. Messenger RNA 261

This book is intended to compare, in reasonable detail, some of the more important experimental systems in which the control of gene expression is studied. These systems consist primarily of studies of the proteins produced by cells, since different proteins are, in general, easily distinguished and monitored. The primary product of all genes is, however, RNA and indeed this is the terminal product of many genes, e.g. those coding for transfer or ribosomal RNA. It is, therefore, crucial to discuss, at least to some extent, the types of RNA found in cells and their relevance to general problems of gene expression. It is a topic which has been very extensively reviewed in the last few years and the reader is referred to other books for a more exhaustive coverage than will be attempted here—Lewin (1974a, b), Gurdon (1974) and Harris (1974).

A. Ribosomal RNA

1. Synthesis and processing of RNA

Three distinct sizes of RNA contribute to the structure of ribosomes. They are 5S, 18S and 28S in eukaryotic cells and 5S, 16S and 23S in prokaryotes. The eukaryotic organization will be discussed here and readers are referred to Lewin (1974a) for a full discussion of the synthesis and assembly of ribosomes and ribosomal RNA in prokaryotes. Of the three species of ribosomal RNA, the two larger are known to be originally combined in a single primary transcriptional product, which is later cleaved and trimmed to yield the 18S and 28S RNA. This primary product sediments at 45S and has a molecular weight of about 14 000. It is first trimmed to a 41S RNA by the removal of a terminal piece and this 41S is then severed to yield one 32S and one 20S section.

Each of these sections is then further trimmed by nuclease activity to yield the final 28S and 18S products which are utilized in the structure of the ribosomes. There is no evidence that the 5S RNA is trimmed down from a larger transcriptional product.

Actual ribosome assembly, that is association of ribosomal RNA with the ribosomal proteins, is chiefly nuclear. The ribosome is itself made up of two particles or subunits, 60S and 40S in eukaryotes, 50S and 30S in prokaryotes, combining to form a single ribosome, sedimenting at about 80S or 70S respectively. It seems likely that the 60S particle contains one molecule of each of the 28S and 5S, and the 40S one of each of the 18S and 5S RNAs, in eukaryotes; the 50S contains a 23S and a 5S, and the 30S one molecule of 16S, in prokaryotes. A large number of protein molecules are found in each ribosome, most being single molecules of separate species. The bacterial ribosome has a total of about 50 protein molecules, but the eukaryotic ribosome may have as many as 80.

2. Function of the ribosome

The number of ribosomes/cell is estimated as 15–20 000 for *E. coli*, 500 000 for a fission yeast (Maclean, 1965) and in the cells of higher organisms they must number many millions. Of the many interesting aspects of ribosome structure and function, two have a special relevance to the subject of this book. One is the general question about polysome structure and the relationship of the polysome to the endoplasmic reticulum and the other is the topic of ribosomal specificity and the possible commitment of some ribosomes to the production of particular proteins.

In relation to the first question, it can be fairly stated that aggregates of ribosomes can be isolated from the cells of bacteria and reticulocytes, which give every indication of being held together by their association with a single molecule of messenger RNA (Kiho and Rich, 1965; Warner *et al.*, 1963).

Combinations of enzymatic digestion and electron microscopy confirm that the link is indeed RNA and addition of messenger RNA fractions to suspensions of single ribosomes can result in polysome formation. However, some important questions remain unanswered in regard to polysomes, not least about the mechanism which drives the ribosome along the messenger RNA or the mRNA through the ribosome. But, perhaps, the area of greatest uncertainty is about the relevance of the polysome concept to the average eukaryotic cell. Most cells of higher animals and plants possess an elaborate endoplasmic reticulum to which a very large percentage of the cellular ribosomes are apparently attached. Soon after the polysome concept was first proposed, papers began to appear in the journals, in which rows and whorls of ribosomes in cell sections were arrowed as polysomes. But since the chances of sectioning a random string of beads in longitudinal section, so that more than three or four adjacent beads are intersected, are very small, it is clear that many of these groups of ribosomes must be sections of large arrays of ribosomes. It is very likely that such arrays are held together not by messenger RNA but by their association with endoplasmic reticulum. The question of the movement of the mRNA and/or the ribosome is then even more acute. Of course, not all active eukaryotic ribosomes are attached to membranes, but many are, and the linkage to the membrane cannot be properly left out of the model of the functional assembly of messenger RNA and ribosomes.

The second question about ribosome function concerns the possible specificity of ribosomes to the production of particular proteins. There is some evidence from bacterial systems that not all ribosomes will translate all mRNA with equal efficiency (Lodish, 1970). It has often been suggested that protein for export is mainly or entirely synthesized by bound ribosomes, while protein for internal use is made on free ribosomes. Evidence appears to be contradictory, but some experiments do reveal that immunoglobulins, which are certainly for export, are made on both free and bound ribosomes. On the other hand, preferential binding of homologous mRNA to ribosomes, as compared to heterologous mRNA binding, has been reported (Naora *et al.*, 1971; Whitelam and Naora, 1972). Certainly, the efficiency with which the ribosomes of frog's eggs and embryos translate injected globin mRNA (Gurdon, 1974) hardly suggests a high level of ribosomal specificity, but it may be that, once committed to a particular mRNA molecule, particular ribosomes might tend to retain the commitment in successive translational runs, even if only for reasons of spatial convenience.

3. Arrangement and expression of genes for ribosomal RNA

As seen in Table 5, all of the genes for the rRNA molecules are present as multiple copies in almost all cells. Actually, *Mycoplasma* has only single copies of its 16S and 22S rRNA gene

(Ryan and Morrowily, 1969). But *E. coli* has six copies of each of the 16S and 23S and between seven and 14 copies of the 5S RNA (Spadari and Ritossa, 1970), while mammals have about 280 copies of each of the 18S and 28S and about 2000 of the 5S (Hatlen and Attardi, 1971; Aloni *et al.*, 1971; Lewin, 1974b). In *E. coli*, the 16S and 23S genes are linked and probably transcribed as a polycistronic RNA which is later broken down to size (Kossman *et al.*, 1971). There is also evidence suggesting that all the ribosomal RNA genes, both the 16 and 23S pairs and the 5S, are clustered into one or two main groups in the genome (Lewin, 1974a for discussion, and Matsubaru *et al.*, 1972) of *E. coli.* Similarly, the 18 and 28S genes are transcribed together in higher organisms, as discussed above, and the numerous gene copies are tightly clustered into one large mass which is often recognizable in the light microscope as the nucleolar organising region of the genome. The anucleolate mutant of *Xenopus laevis* synthesizes no 18 or 28S RNA (Brown and Gurdon, 1964) but continues to synthesize 5S RNA normally. Mutants of *Xenopus* which lack part, but not all, of the nucleolar RNA have been isolated by Knowland and Miller (1970). Similarly bobbed mutants of *Drosophila* lack some or all of the major ribosomal RNA genes (Ritossa and Spiegelman, 1965).

TABLE 5. Numbers of genes coding for transfer and ribosomal RNAs in different organisms (from Gurdon, 1974). Data taken from results obtained by many different authors.

	No. of genes coding for				
Species	tRNA	18S/28S rRNA	5S rRNA	Size of genome	Organism
Bacterium	50	6	7–14	$2 \cdot 8 \times 10^9$ daltons	*E. coli*
Bacterium	42	9–10	4–5	$3 \cdot 9 \times 10^9$	*B. subtilis*
Yeast	320–400	140	–	$1 \cdot 25 \times 10^{10}$	*S. cerevisiae*
Insect	860	180–190	195–230	$1 \cdot 2 \times 10^{11}$	*D. melanogaster*
Frog	1150	450	24,500	$1 \cdot 8 \times 10^{12}$	*X. laevis*
Mammal	1310	280	2000	$3 \cdot 1 \times 10^{12}$	*Hela* (human)

Positioning of the 5S gene copies in the eukaryotic genome is less simple than for the major rRNA genes. They are apparently in a single cluster on chromosome 2 in *Drosophila* (Wimber and Steffensen, 1970), in a single cluster in Man, but in numerous clusters on many chromosomes in *Xenopus* (see discussion in Pilone *et al.*, 1974).

When the sequences of the multiple copies of the rRNA genes are compared, they are found to be extremely and, perhaps, completely homogeneous both in bacteria (Lewin, 1974a) and in *Xenopus* (Brown *et al.*, 1972). Indeed, although all the hundreds of copies of the 18 and 28S genes themselves were identical, not only within one species but also between two different species of *Xenopus,* these authors also found that the spacer DNA region between these genes had diverged in the two species to become widely different. Clearly, some important mechanism is in operation to ensure that copies of the genes do not become different. Such a mechanism of gene matching may also occur in the control of immunoglobulin production (see Section 3A).

It is interesting to examine the available facts about the control of transcription of the rRNA genes. There is good evidence, from experiments on double labelling of developing *Xenopus* eggs and embryos, to show that the 28 and 18S genes are always read at the same relative rate, but that the 5S gene expression is out of phase with them. Indeed, a vast excess of 5S RNA is synthesized in the early oogenesis of *Xenopus laevis* (Mairy and Denis, 1972). There is even evidence that, at least in some species, the 5S genes transcribed in early development are different from those transcribed later (Ford and Southern, 1973). The coordinate synthesis of 18 and 28S RNA no doubt reflects their original transcription into a single polycistronic RNA. It is an open question whether there is any tight control over the transcriptions of the rRNA genes at all and this question will be discussed further in the next section of this chapter.

Another interesting observation is that of Amaldi *et al.* (1973) which indicates that the number of gene copies of the major rRNA genes in 22 different species of amphibians fall into four distinct classes of about 1000, 2000, 4000 and 8000. The observation suggests control of the overall gene number in some relatively simple arithmetic way. A rectification process for the ribosomal cistrons has already been suggested from other data and is discussed in the next section of this chapter.

A recent paper by Honjo and Reeder (1973) indicates that in hybrids between *Xenopus laevis* and *Xenopus mulleri*, the *X. laevis* ribosomal cistrons are preferentially transcribed (as determined by the sequence differences in the non-conserved portions of the precursor RNA), irrespective of which parent is which species. This repression of the *X. mulleri* rRNA cistrons sometimes becomes less pronounced later in development. This is another example of allelic exclusion (see Section 3A) and is, perhaps, most easily explained by production of a specific repressor (perhaps even an ineffective RNA polymerase which blocks the sequence), by the *X. laevis* genome.

4. Amplification and magnification of genes for rRNA

One of the most exciting phenomena discovered in recent years in molecular biology involves the temporary but dramatic increase in the number of gene copies for the major rRNAs in oocytes. During the maturation of the oocyte in many species, including insects, molluscs, fish and amphibians, there is a greatly increased number of nucleoli present—the number may be as high as many thousands. Many of these nucleoli are apparently quite free from the chromosomes, at least in some species, and each contains a core of DNA which conforms in base sequence to the nucleolar DNA block of 18S and 28S gene copies. The phenomenon was described by Gall in 1968 and has been reviewed by Birnstiel *et al.* (1971). Whereas the number of gene copies of the major rRNA in somatic tissues of *Xenopus laevis* is of the order of 2000, in the oocyte this rises to about 2×10^6, involving a thousandfold increase. It must be emphasized that the phenomenon is transient, being limited to the developing egg and very early embryo and is known to occur in only some species. Moreover, no other genes besides the 28S and 18S rRNA are known to be amplified, not even the 5S RNA genes. It has been suggested that a RNA dependent DNA polymerase enzyme (reverse transcriptase) might be involved in the amplification of the DNA (Crippa and Tochinni-Valentini, 1971), but the original experiments have not been satisfactorily substantiated.

Another phenomenon which involves alteration in the number of gene copies of the major ribosomal RNA cistrons is gene magnification (sometimes more generally termed rectification). The main evidence for this process comes from a series of mutants of *Drosophila*, termed bobbed (Ritossa, 1973). This is a recessive mutation which maps in the nucleolar organizer region of the chromosomes, although the overt phenotype by which it was detected involved simply a shortening of the bristles of the cuticle. The mutation has been shown to involve a partial deletion of the block of major ribosomal RNA genes and the number of gene copies left in the mutants may range from 30 up to the normal diploid number of 300 copies. Two aspects of the biology of the bobbed mutation deserve mention. The first is that even a twofold reduction in the numbers of the rRNA genes does not lead to any detectable reduction in the amount of ribosomal RNA synthesized. A second notable point is that some flies which are homozygous for the deletion, produce progeny which show a high proportion of apparently spontaneous reversion to the normal wild type rRNA gene number (thus the terms, rectification or magnification). This return to the wild type dose of rRNA genes is also displayed in the progeny of flies with increased rRNA genes due to genetic duplication of the nucleolar region. So rectification may involve reduction as well as increase.

In terms of gene expression, there are probably two main conclusions to be drawn from these observations on the amplification and rectification of the ribosomal cistrons. One to note is that there is apparently a mechanism available which can correct the number of these gene copies upwards or downwards in order to retain a norm for the species. It is conceivable that this rectification of gene number is also related to the apparent matching of the rRNA gene sequences, which are entirely homogeneous within an organism. The second is that the multiple copies of the ribosomal genes may well be transcriptionally active throughout the life of most cells and actually lack any specific transcriptional regulation. This would help to explain the need for a special amplification process, in times of high demand, for rRNA (see Ashworth, 1971; Gurdon, 1974). But, it has already been pointed out that bobbed mutants which are deficient in rRNA genes may, nevertheless, synthesize normal amounts of rRNA. This would have to be explained in terms of the activity and availability of RNA polymerase enzyme in the tissues examined, i.e. that in the wild type flies the rRNA genes may not always be transcribed at an optimal rate, due to shortage of RNA polymerase.

In addition to these main points, it is also of interest to observe that the 5S RNA genes do not behave in this way. They are not amplified, but, on the other hand, they produce vastly more product early in oogenesis than they do later in the life of the organism (Mairy and Denis, 1972). The 5S genes apparently modulate their activity to the requirements of the 28 and 18S genes, since they are present but untranscribed in anucleolate mutants of *Xenopus* (Gurdon, 1974). 5S RNA is also synthesized during metaphase, a time in the cell cycle when 28S and 18S RNA are not produced (Zylber and Penman, 1971). The 5S RNA genes can, therefore, be viewed as being under normal transcriptional control, as are, for example, the genes for the ribosomal proteins, which also remain silent in the anucleolate mutant of *Xenopus* (Hallberg and Brown, 1969). Attempts to isolate molecules with specific inhibitory powers over the major rRNA genes (Crippa, 1970) must still be regarded as preliminary.

B. Transfer RNA

Transfer RNA is the best understood of the various classes of RNA and the entire nucleotide sequences of several transfer RNA molecules are known. It is also the smallest cellular RNA, sedimenting at about 4S, in both prokaryotic and eukaryotic cells, and varying between 75 and 85 nucleotides in length. The first tRNA to be sequenced was alanine tRNA from yeast (Holley *et al.*, 1965). Each type of tRNA has a distinct sequence and well over twenty types exist in each cell, but all apparently conform to a common pattern and can be fitted into a clover leaf shape as a tertiary structure (see discussion in Lewin, 1974a). Each tRNA molecule has a number of separate active sites, e.g. an amino acid recognition site, the anticodon site recognizing the codon triplet on the messenger molecule, a ribosome attachment site and probably others. At least fifty-six different rRNA species have been isolated from *E. coli* (Muench and Safille, 1968) and it appears that each amino acid has at least two, and may have up to five, different tRNA. Since only 0·06% of the *E. coli* genome hybridizes with transfer RNA (Birnstiel *et al.*, 1972), only about 60 genes must be devoted to these molecules, which might imply only one for each tRNA type. Actually, two identical genes for tyrosine tRNA were identified in *E. coli* (Goodman *et al.*, 1970). The total number of tRNA genes rises to about 750 in *Drosophila* (Ritossa *et al.*, 1965) and about 1300 in the human *Hela* cell (Hatlen and Attardi, 1971). The 4S genes appear to be in a number of clusters, but the clusters are fairly generally distributed throughout the chromosomes.

The subject of the role of tRNA in cell differentiation has been reviewed by Sueoka and Kano-Sueoka (1970). Although it is obviously possible for synthesis and availability of tRNA to operate as a control over protein synthesis at the translational level, little direct evidence supports this speculation. Jacobsen

(1971) has reported that a specific isoacceptor form of tyrosine tRNA functions as an inhibitor of tryptophan pyrrolase in a vermilion mutant of *Drosophila* and perhaps some changes in the pattern of proteins synthesized by maturing cells might reflect changes in the availability of tRNA molecules (Lee and Ingram, 1967). This aspect of tRNA has been previously discussed (Maclean and Jurd, 1972) together with some estimates which indicate that, in many or most cells, tRNA molecules are used more than once.

C. Heterogeneous nuclear RNA

The existence of a species of RNA in the cell nucleus, other than the transfer, messenger and ribosomal RNA also represented in the cytoplasm, finds primary recognition in the publications of Harris (1959) and Harris and Watts (1962), and was later reviewed by Georgiev (1969). This RNA is variously described as heterogeneous, heterodisperse, high molecular weight or simply nuclear RNA, although, of course, all species of RNA may be isolated from nuclei under appropriate conditions. Such a fraction of RNA has been obtained from many tissues, including sea urchin embryos (Aronson, 1972), mouse embryo liver (Williamson *et al.*, 1973) and immature duck erythrocytes (Attardi *et al.*, 1970).

The basic experimental evidence for the existence of this species of RNA is to give culture cells a short burst of radioactively labelled uridine. Most of the label in an actively growing culture will be recovered first as a 45S precursor to ribosomal RNA and later as ribosomal RNA in the ribosomes. But, at the time when most of the label is present in nuclear 45S RNA, some can be recovered in RNA of a wide range of sedimentation values, including molecules much larger than any found in cytoplasmic messenger RNA. Fortunately, the kinetics of labelling of the ribosomal precursor RNA and the heterogeneous nuclear RNA are different (Warner, 1966) and much of the Hn RNA turns over rapidly within the nucleus with a half life of only a few minutes (Soeiro *et al.*, 1968). Even with the differences in uptake kinetics, it is not possible to effectively separate the ribosomal precursor RNA from the Hn RNA of intermediate size. One procedure which has been employed to overcome this difficulty is to expose cells to very low doses of Actinomycin D prior to the labelling procedure. Since ribosomal RNA synthesis is much more sensitive to the inhibitory effects of this drug than is Hn RNA, labelled RNA of intermediate size can then be taken to be Hn RNA also.

The size range of Hn RNA is considerable, probably from 10–100S, although at both ends of this scale sedimentation artefacts may be involved in the S values. Certainly, a substantial proportion of Hn RNA may sediment above 50S and some above 70S, indicating a molecular weight of perhaps $1–15 \times 10^6$ daltons (Greenberg and Perry, 1971). The RNA of mature chicken erythrocytes includes a fraction which sediments heavier than 70S (Maclean and Madgwick, 1973). The electron microscope has provided direct visual evidence for very long RNA molecules within the nucleus (Granboulan and Scherrer, 1969) and as the primary transcriptional product of non-ribosomal DNA.

Shortly after the discovery of Hn RNA in the nucleus, it was viewed as possible precursor of cytoplasmic messenger RNA and indeed the advocates of tandemly repeated gene sequences (e.g. master/slave models of gene arrangement) saw this RNA as a possible repeating sequence of identical mRNA molecules. Others saw them as examples of polycistronic mRNA precursor molecules. To date, however, no evidence has been forthcoming for any Hn RNA being polycistronic, but there is evidence from some careful experiments of Imaizuni *et al.* (1973) that each Hn RNA molecule contains only one identifiable mRNA sequence. These authors used the Hn RNA of erythroid cells as a source of globin message sequences, as also did Williamson *et al.* (1973) and Spohr *et al.* (1974), the animals used being the duck and the mouse respectively. There are three main lines of evidence which now go to suggest a precursor product relationship between Hn RNA and mRNA. They are, (a) that both RNA show a similar response to Actinomycin D, (b) kinetic studies on the addition of poly A sequences to Hn RNA, after transcription, at the 3′ end, also tally with the presence of poly A sequences at the 3′ end of many mRNA molecules and (c) hybridization studies which demonstrate the presence of many mRNA sequences in the Hn RNA fraction. The topic of the relationship between Hn RNA and mRNA is discussed at length by Lewin (1974b).

If Hn RNA molecules include only one sequence of mRNA, what makes up the rest of the molecule? After all, the Hn RNA may be 10 or more times the size of the mRNA sequence. Present evidence suggests that most of the remainder of the molecule is degraded within the nucleus and never translated. It is certainly easy to believe that the remainder of the molecule is made up of the relatively long length of DNA which runs from the promoter sequence to the beginning of the coding triplets of the cistron. This DNA, which probably makes up most of the length of the chromomere of most chromosomes and, certainly, of the giant chromosomes of Diptera (see discussion in Maclean and Hilder, 1975), is interpreted as control sequences by Crick (1971) and as junk DNA by Comings (1972). The discovery that much of the DNA consists of alternating segments of unique sequence and repetitive sequence elements, both in *Xenopus* (Davidson *et al.*, 1973) and in slime moulds (Firtel and Lodish, 1973), surely, adds some backing to this general concept of Hn RNA, especially since Lodish *et al.* (1974) have confirmed that, in slime moulds, the portion of the Hn RNA which is degraded does consist of repeated sequence RNA.

Although Hn RNA is probably a high molecular weight precursor of much of the cytoplasmic mRNA, not all mRNA may be processed in this way. Histone mRNA not only lacks a poly A sequence at its 3′ end, but also is not detectable in the Hn RNA fraction. On the other hand, not all Hn RNA is likely to be even potential in mRNA precursor. A fraction of Hn RNA devoid of added poly A sequence, but including short poly A sequences within its transcribed sequence, has no detectable equivalent product in the cytoplasm (Nakazato *et al.*, 1973).

If this general model of Hn RNA is correct, we are left with some intriguing questions. The two most obvious are, (a) why transcribe so much DNA when it is not wanted for translation and, (b) how is the degradation controlled to ensure preservation of the mRNA and the 3′ end of the Hn RNA molecule. If much of the untranslated portion is control sequence, it is not easy to understand why it should be transcribed, especially if positive control is the general model for eukaryotic gene regulation, since this would assume attachment of a regulatory molecule to the control gene sequence in order to effect transcription. As is discussed further in Chapter 7, however, it seems much more probable that much of the gene regulation of eukaryotes is mediated by negative control, and removal of the repressing molecules would permit transcription of the control gene and movement of the polymerase molecule towards the structural gene sequence. Negative control would, therefore, more easily explain transcription of control gene sequences and later transcription of the structural gene.

Control of the degradation of the Hn RNA in the nucleus is not, at present, understood. Obviously, the simplest model would be the insertion of a particular and constant sequence between the termination of the control genes and the start of the unique sequence, signalling the end of the enzyme degradation. The process of trimming of the Hn RNA seems to operate invariably from the 5′ end, while the conserved 3′ end, where the mRNA sequence is presumed to be, has the poly A attached before release from the nucleus.

It is worth commenting that none of the present evidence on the processing of Hn RNA is suggestive of a nuclear screening of particular mRNA molecules. Such a process, if existent, remains purely speculative.

D. Messenger RNA

The existence of messenger RNA was originally proposed in order to explain the rapid switch of bacterial ribosomes to the production of new protein on induction (Jacob and Monod, 1961). The discovery of an unstable intermediate RNA in phage infected bacteria seemed to fill the bill nicely (Brenner *et al.*, 1961; Gros *et al.*, 1961). Despite some early scepticism, the isolation of relatively pure preparations of specific mRNA for globin (see Section 3C), fibroin (see Section 3F), ovalbumin (see Section 3H), histone (see Section 4H) and some other proteins has proved the authenticity of this molecular species of RNA. An enormous literature has grown up around the subject of mRNA and no attempt at a general review will be made here. The reader is referred to Lewin (1974a,b), Mathews (1973) and Williamson (1972) for a more adequate discussion of the topic. Here the discussion will be confined to a few aspects of mRNA which impinge particularly on gene expression.

1. Characteristics of the molecule

Messenger RNA is a population of molecules which varies greatly from one cell to another, depending on the state of development and differentiation of the cell. In most or all cells, some genes are never transcribed and are, therefore, not represented amongst the mRNA. Within any one cell the mRNA fraction is likely to be heterogeneous, except in certain highly differentiated cells where one mRNA species, say globin or fibroin mRNA, predominates. Some mRNA is also complementary to repeated sequence DNA, but what this DNA codes for is not known (Campo and Bishop, 1974). The histone mRNA is an example of such a fraction. As the sizes of structural genes and their polypeptide products vary in length, so does the messenger RNA, being essentially complementary to the structural gene in terms of base sequence and possessing a triplet of bases for each amino acid to be inserted in the appropriate polypeptide chain. This heterogeneity of size is reflected in the mRNA molecules of, say, *Hela* cells, which will sediment in a range of sizes from 5–30S, with a tendency to form a peak at about 18S (Penman *et al.*, 1968). Such a peak unhappily coincides with the peak of one of the ribosomal RNAs and it is not easy to obtain mRNA fractions completely uncontaminated by rRNA. Various other characteristics of the molecule have been used by various authors to permit identification or isolation of mRNA, but many of these characteristics often hold for only some mRNA or some types of tissue. They include (a) a base composition resembling the total genomic DNA, (b) rapid labelling and fast turnover, (c) sensitivity to ribonuclease, (d) differential sensitivity to Actinomycin D, (e) association with polysomes, (f) hybridization with unique sequence DNA, (g) presence of a tail of poly A residues at the 3′ end and (h) template activity in protein synthesizing systems. There is also some recent evidence (Proudfoot and Brownlee, 1974) which suggests that many or most mRNA molecules have a common sequence of about 52 nucleotides at the 3′ end of the molecule next to the attached poly A sequences. Its function is still unknown.

2. Synthesis and precursors

mRNA is the product of transcription of the structural genes but is often first synthesized as part of a larger precursor molecule, the heterogeneous nuclear RNA (see section above). Discussion of the regulation of transcription will be found in Section 7A of this book. Not all mRNA molecules may take this path, however, since histone mRNA, and probably other species as well, cannot be detected amongst the Hn RNA fraction. The Hn RNA is, apparently, partially degraded in the nucleus to yield the mRNA. Before degradation, however, many Hn RNA molecules receive an addition of some 200 nucleotides of poly adenylic acid at the 3′ end. This tail of poly A residues is believed to be added after the primary synthesis of the Hn RNA molecule, although slime moulds are apparently an exception to this rule, and in these cells the poly A is part of the original transcription product (Lodish *et al.*, 1974). Actually, many of the Hn RNA molecules receive no additional poly A and it is presumed that either these molecules contain no mRNA and are totally degraded in the nucleus, or they contain an mRNA sequence which is selectively rejected in that cell at that time. The fact that the poly A sequence is somehow involved in survival of the mRNA in the nucleus and its shipment to the cytoplasm is suggested by the finding that no mRNA is released to the cytoplasm when poly A synthesis is blocked (Lewin, 1974b).

The next question to be answered is what happens to mRNA after the unwanted parts of the Hn RNA have been degraded? Does it leave the nucleus as mRNA alone, as a ribonucleoprotein or already attached to a ribosome or ribosome precursor? Free mRNA cannot normally be recovered from the cytoplasm of any cell type. At present, it looks very much as if the mRNA is conjugated with one or two protein molecules in the nucleus. There are many reports of cytoplasmic mRNA being recovered by gentle isolation methods in the form of a ribonucleoprotein complex (Blobel, 1973; Jacobs-Lorena and Baglioni, 1972; Henshaw, 1969). At least one of these proteins appears to bind to the poly A sequence (Blobel, 1973) which helps to allay fears that such proteins might be ribosomal proteins acting as initiation or translation factors. Indeed, there is evidence that the proteins may be attached to the poly A of the Hn RNA, before it is trimmed down to yield the mRNA (Lukanidin *et al.*, 1972). There is plenty of evidence that the presence of these proteins is unnecessary for mRNA template activity, so perhaps it is involved in mRNA processing or transport prior to translation. As to the remaining part of our question about attachment of mRNA to ribosomes, current evidence favours attachment in the cytoplasm. That is, that mRNA would leave the nucleus as a ribonucleoprotein complex (RNP) and link up with ribosomes in the cytoplasm (Spohr *et al.*, 1970). But evidence on this point is far from conclusive and the statement is based, largely, on the existence of a free pool of RNP in the cytoplasm, unattached to polysomes. It should be stressed that, although selective breakdown of Hn RNA may occur, perhaps dependent on its possession of poly A sequence, there is at present no evidence for nuclear selection of particular mRNA. We must assume, until facts to the contrary are presented, that all mRNA sequences which are synthesized do eventually leave the nucleus for possible translation in the cytoplasm.

3. Utilization in the cytoplasm

As has been emphasized in the previous section, mRNA is probably released to the cytoplasm as a ribonucleoprotein particle. On its arrival in the cytoplasm, it seems very probable that it is not necessarily immediately utilized. It is known from such experimental systems as *Acetabularia* (see Section 5D) that mRNA must often be present in the cytoplasm for days or even weeks before it is ever translated. Similar evidence emerges from studies on availability and translation of mRNA in oocytes and eggs before and after fertilization (Spirin, 1966). Such masked forms of mRNA have come to be termed informosomes (Spirin, 1969) and the common assumption about such particles is that the protein part of the complex is in some way involved in protecting the mRNA from degradation. Just how and where informosome mRNA is sequestered is still unknown, but it may depend largely on cellular compartmentalization and transport. There are certainly many instances known in which cytoplasmic localization of differentiating molecules exists (see discussion in Gurdon, 1974) and many of these examples may involve informosomes.

The role of the poly A sequences which become attached to the mRNA after transcription remains problematical, although, as has been already pointed out, they may play some part in ensuring survival of selected Hn RNA which contain mRNA sequences. It has been suggested that this tail of poly A sequences might play a part in determining messenger RNA life span by acting as a ticketing system in which one of the poly A sequences was cleared from the end each time the molecule functioned as a template. Certainly, the poly A is not essential to the fidelity of mRNA translation, but Huez *et al.*, (1974) have shown that mRNA, with poly A attached, retains high efficiency as a template for a longer time than the same mRNA deprived of its poly A sequences.

The possible preference of some ribosomes for some species of mRNA has been discussed above under the section on rRNA. It also seems possible that different mRNA molecules may compete for ribosomal binding sites, some being more effectively bound than others. Post transcriptional regulation by selective mRNA translation seems to be a common aspect of cellular differentiation. Another way of achieving the same end result is the operation of a system of selective breakdown of different mRNA molecules. Terada *et al.* (1971) detected a more rapid degradation rate for a 9S mRNA, presumed to code for a nuclear protein, than for a 10S presumptive globin mRNA, in yolk sac erythroid cells.

In general, the half life of mRNA seems to be short in bacteria, i.e. about 20 mins, indicating that most of these molecules are probably only translated once (Forchhammer *et al.*, 1972). These authors also find that the precise half life varies from one end of a polycistronic message to the other, degradation proceeding from the 5′ to the 3′ end. On the other hand, most of the eukaryotic mRNA has a comparatively long half life of hours, days or even longer. The half life of any particular mRNA almost certainly varies with many factors, especially as between one tissue and another.

7. General Concepts of Gene Regulation

A. Chromosome structure and gene masking 266
B. Post-transcriptional control and the role of the cytoplasm 279
C. Hormones and other extracellular factors 283

In this chapter, some of the more important concepts of eukaryotic gene regulation will be discussed. These concepts are not generally applicable to prokaryotic systems, although some of the theories involved do find a factual basis in evidence drawn from bacterial gene regulation. However, prokaryotic gene regulation, factual and theoretical, has been discussed in Chapter 2 and will not be included for discussion here. Theoretical aspects of gene regulation will be taken in three subsections, one on chromosome structure and general transcriptional control, one on post-transcriptional control and the role of the cytoplasm and one on extracellular signal molecules such as hormones and chalones. It is appreciated that these categories are not entirely exclusive, and that there are important aspects of gene regulation which do not fall neatly into these compartments. But these categories should at least render a confusing subject more manageable. The preceding chapters contain a high content of factual information on gene regulation; in this chapter the more general and theoretical aspects will be discussed and some broad conclusions drawn.

A. Chromosome structure and gene masking

Eukaryotic cells utilize a number of mechanisms for regulating the genes available within a differentiated cell and some of these are at the level of the whole chromosome. Thus, the haploidy of germ cells in contrast to the diploid state of the somatic tissues is such a mechanism; this haploid-diploid differentiation of cell lines is utilized in lower plants such as mosses and in Hymenopteran insects, as an extended phase of the life history, or as a differentiation between the sexes. Thus male drone honey bees are entirely haploid while the female workers and queens are entirely diploid (Englemann, 1970). In other organisms, an

aneuploid situation is used to determine sex, as in the fruit fly *Drosophila*, or in man, where the female is double X and the male XY, the female lacking the Y chromosome. A similar use of chromosome reduction to achieve differentiation occurs in such organisms as the parasitic nematode, *Parascaris*, and the fungus gnats, *Sciara*, in which elimination of some chromosomes takes place in the somatic cell lines and only the germ line retains its full chromosome and gene complement. These examples of manipulation of chromosome number in differing tissues or organisms as the parasitic nematode, *Parascaris*, and the fungus the most gross level—by elimination of the gene from the cell. This phenomenon is highly exceptional in animals and plants; much more generally, gene expression is regulated by retention of all or most of the genes and control is operated by regulating their effective part in RNA or protein synthesis. The rest of this chapter will be devoted to discussion of such regulation.

There is now a wealth of evidence to suggest that eukaryotic gene regulation is achieved by two different mechanisms, complementary in action, operating at different levels. These two mechanisms are, first, the long term inactivation of blocks of genes and, second, the relatively short term control over the expression of any single structural gene not included in an inactive block. The two mechanisms are not, in every case, clear cut, examples being found of active genes within inactivated blocks, e.g. in the sex chromosomes of *Drosophila* and Man. However, the tendency for genes to be inactive within the blocks is obvious enough.

1. Inactivated blocks of genes

One of the oldest observations made on chromosomes was that of their variable heteropyknocity (difference in density) following staining of cellular preparations with a variety of dyes. It soon became clear that regions which stained darkly were relatively constant in location and chromosome regions staining in this way were given the term heterochromatin; chromatin which failed to show this intense and early staining, normally the bulk of the cellular chromatin, was termed euchromatin. As genetic maps became more detailed, a correlation became apparent between heterochromatin and genetically inert regions of chromosomes. Unfortunately perhaps, the term heterochromatin has come to be used for genetically inactive areas of chromosomes whether or not these regions appear heteropyknotic after light microscope staining. In particular, parts of chromatin in interphase nuclei, not differentiated into discrete chromosomes, may still appear more or less condensed in the light or electron microscope and although such areas do not appear heteropyknotic at interphase (as does the X chromosome or Barr body in human females), they are sometimes designated as heterochromatin (Frenster, 1969). The justification for this extended use of the word heterochromatin is not historical but functional, depending on the view that condensed chromatin, whether in a heteropyknotic area of a mitotic chromosome or an electron dense area of interphase chromatin within a nucleus, is essentially the same material. To date, no consistent chemical difference between heterochromatin and euchromatin has been detected, in whatever sense the term heterochromatin is used. Eukaryotic chromatin consists of DNA conjugated with the basic protein histone, a variety of associated acid proteins and, perhaps, some RNA. Certainly, despite the early theories of histone and gene repression (Huang and Bonner, 1962), heterochromatin and euchromatin have closely similar histone contents both qualitatively and quantitatively. The more condensed nature of heterochromatin has led a number of biologists to postulate that its molecular organization may be different from that of euchromatin, and in particular, that in these regions a much higher degree of supercoiling of the DNA/protein complex occurs (DuPraw, 1970). This certainly seems possible although, as will be discussed later in this chapter, more recent observations on DNA/histone structure render the idea of supercoiling less likely (Baldwin *et al.*, 1975; Kornberg, 1974) and would imply that highly supercoiled chromatin is less available for transcription than the more extended euchromatin. Of course, not all heterochromatin is necessarily analogous material and scrutiny of the list below will indicate that different factors may be responsible for rendering chromatin condensed and/or inactive.

(a) *Examples of condensed or heterochromatic chromatin*

Centromeric chromatin and other blocks of simple sequence DNA.
B chromosomes of some grasshoppers and maize.
Y chromosomes of males in Man, insects and other groups, and one of the X chromosomes in human female.
Condensed chromatin, as visualized, for example, in light and electron micrographs of chicken erythrocyte nuclei.

Clear differences can be drawn between some of these categories. For example, it is now obvious that centromeric heterochromatin consists of highly reiterated short sequences of DNA (Jones, 1970), while the condensed chromatin of an erythrocyte nucleus is not characterized by the late labelling in the S period as occurs with sex and B chromosome heterochromatin. Out of this somewhat confused picture, three conclusions are presently possible:

1. Eukaryotic cells have the ability to render blocks of chromatin inactive and, although a variety of mechanisms are involved, the end result has common properties of condensation and inactivity. Even active genes translocated near to heterochromatin may be inactivated or modified in their expression (Becker, 1966).
2. Condensed chromatin blocks are more or less permanently inactivated. Centromeric regions are obviously permanent, the Barr body representing the inactivated X chromosome rather less so (since either X chromosome may be inactivated in neighbouring cells) and the condensed chromatin of the inactive nucleus being relatively impermanent since it is open to reversal when introduced into a heterokaryon situation (Harris, 1967).
3. With the exception of the centromeric heterochromatin, which differs from other chromatin in the character of its DNA sequences, most condensed chromatin appears to differ from euchromatin only in its non-histone protein content (Ruiz-Carrillo *et al.*, 1974; Hilder *et al.*, 1975) and in the acetylation of its histones (Ruiz-Carrillo *et al.*, 1974).

2. Repeated DNA sequences

All eukaryotic cells contain some DNA sequences which are repeated, but there is little evidence that this is frequently a device for controlling gene expression. In other words, there is no good evidence to date to show that, where multiple copies of a gene exist, the number of these copies expressed is modified in accordance with the needs of the cell (gene amplification and rectification are exceptions to this statement, see Section 6A). For this reason, although a short discussion is included here on repeated sequences, it is only a brief outline of the facts at present known. The reviews of Walker (1971) and Flamm (1971) provide much fuller information. Several distinct kinds of repetitious gene sequences occur in eukaryotic cells.

(a) *Highly repetitive sequences*

This type of DNA has already been mentioned in this chapter as characteristic of the centromere. Some of these sequences are also found in other chromosomal locations. It accounts for between one and 30% of the total DNA content of an organism and the sequences appear to be too short to function as normal cistrons. Probably, this DNA fulfils chiefly non-genetic functions and is not transcribed.

(b) *Ribosomal and transfer RNA genes*

As in most prokaryotes, these genes are present in eukaryotic cells as multiple copies, the number of copies being numbered in thousands for some organisms. In contrast to the situation in prokaryotes, the ribosomal and transfer RNA genes are separate in higher cells, the ribosomal genes being clustered into the nucleolar DNA area and the transfer and 5S ribosomal genes, being in separate clusters, concentrated at the ends of chromosomes.

(c) *Other repeated sequences*

Quite apart from the highly repetitive DNA sequences associated with the centromere and the sequences coding for ribosomal and transfer RNA, there is evidence for other sequences being present as multiple copies. Even a glance at the sheer amount of DNA in the eukaryotic genome suggests such a phenomenon. Yet, to date, the evidence for any one protein being coded by multiple gene copies is poor, or at least conflicting. Thus, for haemoglobin, there are reports suggestive of multiple copies (Sanchez de Jimenez *et al.*, 1971) and others excluding anything more than six (Bishop *et al.*, 1972) while, in the case of silk protein, the only evidence is against multiple gene copies (see Section 3F). Only in the case of histone is there satisfactory evidence for multiple copies (Kedes and Birnstiel, 1971) although here the precise number of protein species involved is not known. One of the objections to multiple gene copies of structural genes is that it would render the protein product rather resistant to mutational change, whereas the cistrons for, say, the human α, β, γ and δ globins obviously do mutate readily, as evidenced by the abnormal haemoglobins detected in blood typing laboratories. Attempts to meet this objection have most frequently involved some sort of master-slave model for the gene copies postulated, assuming that all but one of the copies are slave copies, regularly matched with the master copy and so faithfully accepting any mutational change in the master gene. This idea was originally proposed by Callan and Lloyd (1960) and has gained some theoretical acceptance, but also involves many difficulties (see discussion in Comings, 1972 and Maclean, 1975).

Although some of the moderately repeated DNA of the eukaryotic genome may indeed be accounted for by multiple copies of structural genes, such as histone, much cannot be assigned in that way. It now seems much more probable that much of this DNA consists of fairly short sequences moderately repeated in the genome and that groups of these sequences are generally interspersed with single copies of the structural genes throughout the genome (Davidson *et al.*, 1973; Firtel and Lodish, 1973). The most probable role for such DNA is that it consists of control sequences responsible for regulating the complex programme of transcription involved in the cell cycle and cell differentiation as well as DNA specialized for the condensation of the chromomere. This topic is further discussed on p. 277.

(d) *Gene amplification* (see also Section 6A)

Gene amplification is a phenomenon known to occur in only a few organisms during early development and occurs when the number of copies of the ribosomal genes is temporarily elevated. The degree of amplification is large, the ribosomal gene copies of *Xenopus laevis* being raised from 1500 to about two million in the oocyte (Birnstiel *et al.*, 1970). There is no doubt that gene amplification is a device which has evolved in order to greatly modify normal gene expression so as to meet the needs of an exceptional cellular situation. Besides the amphibian *Xenopus*, the process is known to occur also in the water beetle, *Dytiscus* (Gall *et al.*, 1969), and the house cricket, *Acheta* (Lima-de-Faria *et al.*, 1969), and probably in many other species.

It might be legitimate to use the term amplification to describe the local increase in the number of copies of mitochondrial DNA which takes place when these organelles proliferate in certain cell types. The term is not normally used to cover this process and the subject of mitochondrial DNA will be taken up in the next section in this chapter (The cytoplasm).

Not to be confused with heterochromatin, but nevertheless a striking example of comparatively condensed chromatin, is the chromomeric chromatin of lamp brush chromosomes and giant polytene chromosomes (see discussion in Maclean and Hilder, 1975). In both of these chromosomes, most of the DNA—up to 95% in the latter case—is packed into the chromomeric section. Of course, the loops of the lampbrush chromosome are to be seen as an extension of the chromomere, analogous to the puffs of the polytene chromosome. The truly interchromomeric chromatin runs between the chromomeres as interband chromatin and appears to be permanently uncondensed (it may even be permanently untranscribed). Perhaps the most attractive theory of eukaryote chromosome structure which can usefully be advanced is that the structural gene lies within the chromomere, perhaps associated with a series of control gene sequences, and that when transcription is to take place, most or all of the chromomere is unravelled and looped out. It also seems probable that much of the chromomeric DNA is specialized for the process of condensation, since this is clearly an important aspect of eukaryotic gene regulation.

This concept of chromosome structure, by no means proven or universally accepted, none-the-less squares with many of the known facts. If it is broadly correct, it links the condensation and decondensation of the chromomere—or part of it—with the activity of individual genes.

3. Controlled transcription of single genes

The evidence that cellular differentiation depends upon the controlled expression of the structural genes is now overwhelming. Yet understanding of the mechanisms which operate this control remains relatively obscure. In Chapter 2, it was possible to conclude that a large part of the bacterial genome functioned in distinct units termed operons, each relatively independent and sensitive to specific cellular molecules. Operons are not, however, a feature of the eukaryotic genome. Some operon-like units have been identified in fungal systems, e.g. controlling enzymes associated with galactose metabolism, but these can best be viewed as an evolutionary carry-over from bacterial gene regulation. No sound evidence is available for an operon unit occurring in the genome of a higher animal or plant. How then is the genomic expression manipulated? The model which has gained greatest theoretical favour owes its inspiration to bacterial regulation, but assumes that in the eukaryotic system only one cistron would normally occur in the transcription unit but a series of regulatory and promoter genes would be associated with each cistron. The theories of Georgiev (1969) and Britten and Davidson (1969) run along these lines. Clearly, some of the evidence listed as suggestive of master-slave sequences could equally well support a transcriptional model where a large part of a particular complementation group was made up of regulatory sequences. We have already suggested that the chromomere constitutes such a genetic complex, with the additional proviso that much of the chromomeric DNA between the promoter and the gene is devoted to specific condensation.

Of course, the greatest difference between the DNA of prokaryotes and eukaryotes is that the former is relatively naked while the latter is conjugated to a large mass of histone. For this reason, it seemed reasonable to suppose that the histone itself was responsible for regulating the eukaryotic genome and early experiments lent weight to this idea (Allfrey *et al.*, 1964). However, as knowledge of histone increased, faith in its ability to regulate specific gene activity diminished and there is now rather strong evidence that the histones of different tissues from the same organism are qualitatively and quantitatively similar or identical.

The relationship between histone and DNA has until recently remained largely unknown, although it was generally assumed that the histone fitted in or on to one of the grooves of the DNA helix. Some recent evidence (see Section 4H) now renders it much more likely that the DNA is fitted round a histone complex, made up of two different histone tetramers, $(F_2A_1)_2(F_3)_2$ or $(F_2A_2)_2(F_2B)_2$. The fifth histone type F_1, which is known to have very different properties from the other four, probably acts as a cross link between the histone/DNA beads in condensation of the chromatin (Baldwin *et al.*, 1975). Whether this model of DNA/histone is generally applicable to all or even most eukaryotic chromatin is quite unknown. It would certainly be pleasing to find that it applied only to chromomeric chromatin and that intrachromomeric chromatin, which is always extended, lacked this histone association. In any event, it is at least probable that the chromomeric DNA is complexed to histone in this way and that decondensation of this chromatin involves breaking the F_1 cross links between the chromatin beads. Whether this decondensation might precede or follow on from specific gene activation is difficult to forecast or perhaps, at a level of the single structural gene, the two events are not related. The really crucial question which is raised by this model of chromatin organization is whether it should be seen as explaining (a) the gross level of genetical control, the conversion of heterochromatin or other highly condensed blocks of chromatin to a euchromatic form, and *vice versa* or (b) the finer level of genetical control involving the transcription of a single structural gene and perhaps its related control sequences. Since the activated chromomere apparently decondenses to allow synthesis of an Hn RNA molecule, the presumptive mRNA precursor, it may be that an alteration of the packing of the histone tetramers one with another would explain this.

In terms of specific regulatory molecules, the emphasis has now moved to the other constituents of chromatin acid protein and RNA and many authors invoke these molecules as responsible for specific gene regulation (Stein *et al.*, 1972). Thus, MacGillivray *et al.* (1972) and Paul (1971) argue that acid protein has the function of altering the DNA/histone association and permitting temporary unmasking of the gene. These ideas owe a great deal to the papers of Frenster (1965). Paul has not been able to rule out a low level of RNA contamination in his acid protein preparation and Huang and Huang (1968) argue that the RNA is responsible for the observed specificity of transcriptional regulation. Alternatively, the effective molecular complex might be a ribonucleoprotein. In any event, histone is apparently not responsible for the fine control of the transcription of particular cistrons in eukaryotes. The sites sensitive to the unmasking molecules are presumably some of those postulated by Georgiev (1969), Britten and Davidson (1969) and Davidson and Britten (1973) as regulatory for individual genes.

As mentioned in the chapter on prokaryotic gene expression, the lac repressor molecule is a tetrameric protein and so the notion that the molecules responsible for eukaryotic gene regulation might be non-histone nuclear proteins is not without support.

The studies of Axel *et al.* (1973) have demonstrated that liver chromatin does not transcribe globin DNA sequences but reticulocyte chromatin does (see discussion in Section 3C). This confirms the widely held view that differentiated function is accomplished by specific gene masking. Moreover, in reconstitution experiments, Barrett *et al.* (1974) have demonstrated that non-histone proteins from reticulocytes (but not from liver) will activate the transcription of globin genes. As discussed in Section 4H, non-histone nuclear protein is something of a rag-bag, and specific regulatory proteins may comprise only a small part of the total population. But there does now seem to be a reasonable body of evidence that this protein fraction includes regulatory molecules. There is also circumstantial evidence supporting the proposal that phosphorylation and/or acetylation of histones (Ruiz-Carrillo *et al.*, 1974) and non histone proteins (Karn *et al.*, 1973) may greatly influence or even determine their function as regulatory molecules.

This view of transcriptional regulation is largely based on the assumption that most or all gene control in eukaryotes is positive (see discussion in chapter on fungi), and that regulatory non-histone proteins act as inducers. But if Hn RNA is truly a transcript of the structural gene, plus its preceding control sequences, and activation involves transcription of the whole (or much of) a chromomere, then negative control would be a more acceptable mechanism. In that case the non-histone proteins would be expected to include both repressor molecules and inducer molecules, the latter having specificity not for any DNA sequence but for the sequence or shape of the appropriate repressor molecule already located on the DNA.

An interesting proposal has recently been made by Holliday and Pugh (1975) which relates both to the activation of individual genes and to the programming of gene expression in development discussed on p. 278. Their proposal is that some regulatory molecules might function as degradative restriction enzymes on the one hand and modifying enzymes on the other, the latter being effective by the methylation of DNA bases, so rendering the DNA inaccessible to the restriction enzymes. There is evidence from prokaryotes that both processes occur, particularly in axially symmetrical (palindromic) sequences. Such DNA modification would not only help to explain the paramutation phenomena revealed in such situations as transdetermination in *Drosophila* imaginal discs (see Section 5F), nuclear totipotency after transplantation (see Section 4E) and the unstable genetic elements of maize (see Section 5G), but, by assuming a set probability of modification, it is also possible to use such a mechanism to explain fixed relationships between the differentiated state of a cell and the number of divisions through which it has proceeded.

The proposal of Holliday and Pugh is still dependent on gene regulation by non-histone proteins, but in their model the proteins would actually alter the DNA sequence rather than simply impede transcription by their attachment to regulatory sequences. It is, obviously, a mechanism to bear in mind, but its importance must clearly depend on stronger experimental evidence.

4. The use of BUdR (5-Bromodeoxyuridine)

In recent years, a promising new chemical tool in studying gene expression has been discovered—the thymidine analog 5-Bromodeoxyuridine (BUdR). A discussion of its effects and significance will be found in Holtzer *et al.* (1972), Rutter *et al.* (1973) and Davidson and Bick (1973). The compound is found to have little or no effect on fully differentiated cells, although it often has profound and dramatic effects on cells prior to or during differentiation. What is really remarkable is that the effects of the drug seem to be specific for particular proteins, often for the specialized proteins of differentiated cells, e.g. globin in reticulocytes or tyrosine aminotransferase (TAT) in liver, rather than for the proteins of more general use and distribution (Miura and Wilt, 1971; Stellwagen and Tomkins, 1971). On the other hand, negative effects with BUdR exposure are reported for α amylase in parotid gland (Russer and Tsaner, 1974) and some cells seem to show specific stimulation of the synthesis of certain proteins, e.g. alkaline phosphatase synthesis in mouse mammary carcinoma cells (Koyama and Ono, 1972).

The mechanism of action of BUdR remains obscure, although its effect on genes with a high AT content is likely to be maximal on account of its generalized incorporation into DNA as a thymidine analogue. But in any event, if its early promise of having specific effects on only a few key proteins is maintained and substantiated, there are good grounds for thinking that it may provide a handle to one aspect of the control of gene expression.

5. Integration of gene expression in differentiation

In most eukaryotic cells differentiation involves selective gene expression and many of the experimental systems which we have discussed in earlier chapters involve the transcription of whole batteries of different genes. Just how the genetic control of differentiation is accomplished is a fascinating question. Although many authors have hopefully assumed that studies on the activity of one or two genes would inevitably reveal some inkling of how the whole process worked, others have seen best to take a broader view and examine the genetic control of, say, liver

tissue or the intact slime mould. It remains arguable which approach has taught us most. Undoubtedly, both have made a contribution.

In the absence of detailed knowledge one is free to theorize, and a number of theoretical attempts at scaling the hurdle of gene regulation in differentiation have been made. The best known examples are those of Georgiev (1969, 1972), Crick (1971), MacGillivaray *et al.* (1972), Britten and Davidson (1969) and Davidson and Britten (1973). All of these models owe much of their basis to knowledge of bacterial operons and indeed many of the models are fairly similar to one another. A common feature to most is the ability to accomplish co-ordinate control of widely separated genes, all needed as transcriptionally active in a particular differentiated cell. The models assume that control genes would be needed in two forms, some sensitive to extracellular effector molecules such as hormones (sensor genes in the Britten/Davidson terminology and some sensitive to the regulatory products of other genes (receptor sequence in the Britten/Davidson terminology). The emphasis in the Britten/Davidson model tends to be on positive control, with the regulatory genes probably untranscribed, while the Georgiev model favours negative control sequences and ultimate transcription of the derepressed control sequences, in addition to the structural gene. The evidence of Hn RNA as a primary transcriptional product certainly favours Georgiev's model, especially since there is some evidence that the major part of the Hn RNA consists of short sequence RNA which may well constitute the transcriptional product of control gene sequences. The findings of Lodish *et al.* (1974), that the degraded portion of Hn RNA of slime moulds is indeed made up of short sequences, certainly argue in favour of this model. It is also interesting to find a short Hn RNA together with a small amount of interspersed repeated sequence DNA in simple organisms such as slime moulds. Backing for the general concept of interspersed single structural genes and numerous short control gene sequences comes from the DNA hybridization studies of slime mould DNA (Firtel and Lodish, 1973).

The particular emphasis in Crick's model (Crick, 1971) is (a) that a chromomere, consisting of a band plus interband of a *Drosophila* polytene chromosome, might consist of a concentration of control sequences in the band region and the structural gene in the interband (I have already discussed the weaknesses of this model (Maclean and Hilder, 1975), and in particular the strong evidence that the structural gene is located in the band and not the interband), and (b) that the control sequences might be single stranded and be recognized by their three dimensional form, as originally suggested by Gierer (1966). As discussed in Chapter 2 on prokaryotic regulation, the lack of similarity in the base sequences of *E. coli* promoter regions (Sekiya and Khorana, 1974) favours a recognition by the polymerase molecule which might be based on the tertiary structure of single stranded loops of the promoter gene. Although there is at present no good evidence to support the second aspect of Crick's suggestion, it still stands as a possible model. Some general aspects of the problem of nucleic acid/protein interaction depending on symmetry and tertiary structure are discussed by Sobell (1974). Certainly, palindromic regions of axial symmetry appear to exist in eukaryotic DNA (Wilson and Thomas, 1974) as well as in prokaryotes.

It should be pointed out that invoking tertiary structure for control gene recognition implies either that such sequences would not be transcribed, as in positive control, or would lose their three dimensional structure or permit transcription in some views of negative control. But it is highly likely that much of the genome is never transcribed and such sequences might well help to explain the large amount of untranscribed DNA. In particular, there often seems to be a discrepancy between the length of a chromomere, and the comparatively short length of even the larger Hn RNA molecules. It cannot be discussed here, but it should be pointed out, that, although not all sequences are transcribed, all are replicated. Since replication and transcription are not unrelated processes (see Maclean, 1973), it is also worth remembering, in discussing theories of positive and negative control, that the same protein can fulfil both the functions of inducer and of repressor, depending on the DNA sequence. Such a protein has been found to mediate control in the arabinose operon in *E. coli* (Englesberg *et al.*, 1969).

One aspect of gene regulation in differentiation which I would like to stress further is the probable existence of genes primarily controlling the entire transcriptional programme of any one differentiated cell. The number of different types of differentiated cell in even the most complex organism is very limited and probably of the order of 200. This implies that the task of explaining the genetic control of differentiation is not necessarily very complicated and may not be very different from designing an automatic washing machine with a choice of 200 different programmes. Once the programme choice has been made (what embryologists call determination—see Section 5F, imaginal discs), the programme itself is fixed to select the expression of different genes at different times. On this simplistic model, the existence of up to 200 "tissue master genes" would be predicted, each one responsible for determining and controlling a genetic programme for a particular differentiated cell type. Rather similar genes are visualized by Davidson and Britten (1973) and styled integrator genes and these authors stress their dependence on and sensitivity to the effector/sensor gene interaction, effectors being the external signals such as hormones. Would the tissue master genes necessarily be sensitive to such influences? Surely, a more favourable idea would be that such sensitivity is a feature of a lower stage in the cascade of regulation, with the tissue master gene simply being responsible for determining cell type. Of course, it is apparently possible for determined cells, e.g. imaginal disc cells, or even differentiated cells, e.g. carrot phloem parenchyma (Section 5G), or *Hydra* cells (Section 5E) to change their commitment in response to a persistent external signal. These situations do seem to me to be highly unusual, however, and by and large the differentiated and even the determined cell inflexibly retains the original slot of its genetic programme.

B. Post-transcriptional control and the role of the cytoplasm

Two slightly separate topics are brought together in this section. Most, but not all, post-transcriptional control is cytoplasmic. The cytoplasm also has important influences on cellular gene expression quite apart from post-transcriptional regulation, particularly because the cytoplasm possesses genes of its own. It will, therefore, be necessary to discuss the topics separately while indicating where they overlap.

In this section, the term chosen to indicate the level of control is post-transcriptional rather than translational, a term which enjoys common usage. There are two objections to translational control as an appropriate term. The first is that little evidence exists for control being exerted during the course of translation and the second that the term is too narrow, since it is apt to exclude control occurring after transcription but much prior to actual translation or after translation itself. Discussion of most aspects of post-transcriptional control has been covered in Chapter 6 and, here, we will discuss only the relatively late post translational stages.

1. Regulation after translation

Even prior to the release of the completed polypeptide by the ribosome, some further control over the free cytoplasmic protein is exercised. This is accomplished in the case of proteins with a quaternary structure by one subunit affecting the release of another subunit from the cytoplasm. Thus, in the case of haemoglobin synthesis, the rate of release (and, following from this, the rate of synthesis) of the β chain is regulated by the availability of free α chains. A small pool of free α chains is maintained and β chains are only released from their polysomes when they can form dimers with the α chains (Fischer *et al.*, 1968, and further discussion in Review by Maclean and Jurd, 1972). Slight differences in the rate of translation of the α and β chains have also been observed in haemoglobin synthesis (Hunt *et al.*, 1969), suggesting that some regulation might operate by differences in the translational rate for different messenger RNA.

After the completed polypeptide chains have been released from the ribosomes and the appropriate sister monomers have combined to form the complex protein, most of the regulation of gene expression is already complete. But, to the extent that the differentiated cell is the ultimate expression of the genes, some further cytoplasmic effects are relevant to the present topic. These effects involve regulation of the population of proteins present in the cytoplasm at any one time. Processes as diverse as controlled preferential enzymic degradation of certain proteins (Halvorson *et al.*, 1971) or the sequestration of particular proteins in an insoluble matrix (Wright *et al.*, 1968) contribute to such regulation and even the controlled loss or export of protein through the cell membrane must be seen as an important regulatory mechanism governing effective enzymic levels.

2. Modification of nuclear gene activity by the cytoplasm

Although at one time it was customary to think of the nucleus as imposing on the cytoplasm the instructions of the genes, it is now clear that the cytoplasm profoundly affects the activity of the nuclear genes. The experiments on nuclear transfer discussed in

Chapter 4 fully demonstrate this truth. Moreover, since both the histone and acid protein which occur in the chromatin of eukaryotic cells are manufactured in the cytoplasm, it follows that nuclear activity is very dependent on cytoplasmic co-operation. Since bacterial apo-regulator molecules are proteins, it seems likely that equivalent eukaryotic regulatory molecules will also be proteins and probably some are recovered as nuclear non-histone proteins. Manipulation of nuclear gene activity by cytoplasm is presumably mediated by the nuclear membrane, just as the cell membrane controls the entry of molecules into the cell cytoplasm. The findings of Kroeger (1966), that polytene chromosome puffing pattern is affected by the nuclear concentration of certain ions, certainly suggests that the free availability of such ions in the cytoplasm must rank as an important measure of cytoplasmic control over selective gene activity. It is disappointing that, in my own laboratory, we have not found variation in ionic concentration an effective activator of erythrocyte chromatin (Hilder and Maclean, 1974).

3. Cytoplasmic DNA—mitochondria and chloroplasts

Since the cytoplasm of almost all higher cells includes organelles which possess their own DNA complement, the cytoplasm has a genetic role of its own in the expression of these cytoplasmic genes. Full discussion of this topic is not to be included in this book and the reader is referred to the work of Sager (1972), but some consideration to the problem is given in Section 5H. Besides mitochondria and chloroplasts, various other sources of cytoplasmic DNA have been cited in the literature. They include centrioles, basal bodies of cilia (Randall and Disbury, 1965), i-somes (Bell, 1971) and yolk platelets in embryos (Brachet and Ficq, 1965). However, unlike mitochondria and chloroplasts, there is, to date, no compelling evidence to suggest that any of these bodies or organelles possess their own unique fraction of DNA. On the other hand, there is no longer any doubt that both mitochondria and chloroplasts do possess such a DNA fraction and it has now been extracted, purified and extensively studied.

For the purposes of this book, it is not necessary to discuss the now strong circumstantial evidence linking mitochondria with bacteria and chloroplasts with blue-green algae. But it is appropriate to mention that such a relationship is suggested even by parallel methods of gene regulation—the DNA is not conjugated to histone in either case.

What is of particular relevance to cellular genic regulation is the degree of autonomy enjoyed by mitochondria and chloroplasts and their contribution to the differentiated expression of the host cell. First, with regard to autonomy, the following statements can now be made with confidence, (a) mitochondrial DNA is distinct from nuclear DNA and contains unique sequences of its own (Dawid, 1972), (b) mitochondria and chloroplasts are inherited cytoplasmically and, in some cases, more than one population of these organelles may coexist in the same cell (e.g. *Pelargonium*), (c) DNA replication of these organelles is not in phase with the cellular S phase and (d) neither in mitochondria nor chloroplasts is autonomy complete, since many proteins essential to both organelles are coded in the nuclear genes. It is, therefore, correct to state that both of these cytoplasmic particles possess only partial autonomy within the cell.

With regard to the influence on differentiation and final gene expression, the role of both chloroplasts and mitochondria is critical. Cells with defective mitochondria or chloroplasts form distinct cellular strains, e.g. petites in yeast (see Jinks, 1964), and form variegated and white forms of plants with partial or complete lack of chlorophyll in the chloroplast. What the biologist must now strive to understand is a differentiating genetic system (the mitochondrion or chloroplast) within a differentiating genetic system (the animal or plant cell). Since nuclear genes are necessary for the survival and activity of these organelles, the question also arises as to whether products of the mitochondrial or chloroplast genome influence nuclear gene activity. They certainly do influence cytoplasmic gene expression, as evidenced by the nature of the petite mutant in budding yeast—such mutants, induced readily by acriflavine, are mutants of the mitochondrial genome. They may be recognized by lack of cytochrome enzyme activity and yet the genes for the cytochrome enzymes are lodged in the nuclear genome (Jinks, 1964).

A final paragraph in this section must be devoted to some discussion of a type of cellular inheritance which seems to be almost independent of the nuclear or cytoplasmic genome. This phenomenon involves the use of pre-existing cytoplasmic structures as templates for the production of new structures of the same sort. The murein layer of the bacterial cell wall acts in this way (see discussion in Stanier *et al.*, 1971) and removal of this layer by the action of penicillin may render some bacteria permanently devoid of cell wall—the L forms.

An analogous molecular mechanism operates in the elaboration of the polysaccharide glycogen in eukaryotic cells, the presence of glycogen primer proving essential for the synthesis of further lengths of polymer. This principle of the employment of a pre-existing structural template also applies to the function of two related cellular structures, centrioles and basal bodies or kinetosomes. Contrary to earlier reports, there is now little doubt that neither particle possesses its own DNA (Younger *et al.*, 1972; Flavell and Jones, 1972). Centriole replication continues to pose problems, but the mechanisms of kinetosome replication are somewhat clearer. This follows from their activity in ciliated Protozoa, where the cellular cortex contains many kinetosomes arranged in characteristic rows or kinetics. The fascinating inheritance of aberrations in the cortical arrangement of these kinetics is discussed in Section 5C.

C. Hormones and other extracellular factors

Gene activity is not only influenced by nuclear and cytoplasmic molecules but also by extracellular factors which affect the cell surface or enter the cell and induce intracellular changes. Of course, even simple ions and metabolites in a medium will profoundly affect cellular genetic regulation, but in multicellular organisms careful control over the immediate extracellular milieu is normally maintained. In such a situation, the cell responds dramatically to circulating molecules whose function is to evoke specific cellular and tissue responses. Such substances are loosely termed hormones, both in plants and animals. In addition, certain phenomena concerned with growth and cell division in particular animal organs indicate a cellular response to circulating factors apparently released by the tissue itself. Such factors have been termed chalones and these will receive some brief mention in this section. Cellular responses to such substances as phytohaemagglutinin will also be discussed.

1. Animal hormones

The hormones of animals can be subdivided into steroids and polypeptide hormones. Steroids, which include oestrogen, androgen, aldosterone, progesterone and corticosteroids in mammals and ecdysone in insects, are small molecules with a tetracyclic nucleus. They are related to other important biological molecules such as cholesterol. The small size of the steroid molecule presumably facilitates its entry into the target cell and certainly the mode of action of steroid hormones, contrary to that of the polypeptide hormones, involves an intracellular activity.

Non-steroid animal hormones are chiefly polypeptides, although adrenaline, thyroxine and histamine are amino acids or amino acid derivatives. With the exception of these last named molecules, polypeptide hormones are relatively large, insulin having a molecular weight of 6000 and STH (somatotropic hormone) 48 000. It is, therefore, not surprising to find that the polypeptide hormones do not normally enter target cells, but evoke a response by activity at the cell surface.

The mechanisms of action of steroids and polypeptide hormones are now known to be widely different and current theories about both mechanisms have profound inferences for the subject of control of gene expression.

Polypeptide hormones, as has been indicated, operate at the cell surface of the target cell. However, the cellular response to this surface activity is often speedy and complex and it is, therefore, apparent that the information available at the cell surface is rapidly transmitted to the cytoplasmic enzyme pathways. The pioneering work of Sutherland and his collaborators in Tennessee (Butcher *et al.*, 1972) has elucidated that a second messenger system operates in the cell, following the surface binding of a polypeptide hormone molecule. This second messenger, now identified as the cyclic AMP molecule (adenosine 3′, 5′-monophosphate) is responsible for effecting the real cellular response to the hormone, namely alterations in enzyme levels within the cell. There is now evidence that cyclic AMP operates primarily by an allosteric interaction with phosphoprotein kinases (Corbin *et al.*, 1970). Many, but not all polypeptide hormones, induce an increase in cellular cyclic AMP by affecting the activity of the adenyl cyclase enzyme at the cell surface. However, it is clear from reference to Table 6 that not all such hormones induce an *increase* in cellular cyclic AMP. Some hormones, such as insulin, induce a decrease in levels of cyclic AMP and others, such as catecholamines, may operate in differing ways by inducing either an increase or a decrease. Polypeptide hormones are thus believed to affect many different cellular enzymic pathways and, in so doing, considerably modify the instructions of the genes and the cellular expression of these instructions.

TABLE 6. Hormone actions mediated by changes in levels of cyclic AMP. ↑ = increase in cyclic AMP level and ↓ = decrease in cyclic AMP level (from Butcher *et al.*, 1972).

Hormone	Tissue	Effect[b]
	Increased cyclic AMP levels	
Adrenocorticotropic hormone	Adrenal cortex	↑ Steroidogenesis
	Fat (rat)[c]	↑ Lipolysis
Luteinizing hormone	Corpus luteum, ovary, testis	↑ Steroidogenesis
	Fat	↑ Lipolysis
Catecholamines	Fat	↑ Lipolysis
	Liver	↑ Glycogenolysis, ↑ gluconeogenesis
	Skeletal muscle	↑ Glycogenolysis
	Heart	↑ Inotropic effect
	Salivary gland	↑ Amylase secretion
	Uterus	Relaxation
Glucagon	Liver	↑ Glycogenolysis, ↑ gluconeogenesis, ↑ induction of enzymes
	Fat	↑ Lipolysis
	Pancreatic β-cells	↑ Insulin release
	Heart	↑ Inotropic effect
Thyroid stimulating hormone	Thyroid	↑ Thyroid hormone release
	Fat	↑ Lipolysis
Melanocyte stimulating hormone	Dorsal frog skin	↑ Darkening
Parathyroid hormone	Kidney	↑ Phosphaturea
	Bone	↑ Ca^{2+} resorption
Vasopressin	Toad bladder, renal medulla	↑ Permeability
Hypothalamic releasing factors	Adenohypophysis	↑ Release of trophic hormones
Prostaglandins	Platelets	↓ Aggregation
	Thyroid	↑ Thyroid hormone release
	Adenohypophysis	↑ Release of trophic hormones
	Decreased cyclic AMP levels	
Insulin	Fat	↓ Lipolysis
	Liver	↓ Glycogenolysis, gluconeogenesis
Prostaglandins	Fat	↓ Lipolysis
	Toad bladder	↓ Permeability
Catecholamines (α-adrenergic stimuli)	Frog-skin	↓ Darkening
	Pancreas	↓ Insulin release
	Platelets	↓ Aggregation
Melatonin	Frog skin	↓ Darkening

Steroid hormones, in contrast to the polypeptide hormones, pass through the cell membrane and act within the target cell. Indeed, there is mounting evidence that the site of action of the steroid molecule is the nuclear chromatin and perhaps even the gene. The steroid molecule is probably conducted to the chromatin by a carrier protein molecule (Jensen *et al.*, 1971). One or two separate lines of evidence now combine to suggest a chromatin or genic level of steroid action. The first is the finding of O'Malley *et al.* (1972) that, after chick oviduct has been stimulated with oestrogen or progesterone, novel species of nuclear RNA can be detected. A second is the observation that ecdysome will evoke very specific gene responses in the giant chromosomes of *Diptera* (Berendes, 1971). However, some major problems relating to steroid hormone action remain. Cellular responses to any one steroid hormone are often variable and complex and the levels of circulating steroids hardly suggest a level of activity comparable to the specific gene derepression known for bacterial operons. Until more is known about the nature of eukaryotic chromatin, the action of the steroid hormone on the animal cell will probably remain unclear.

2. Plant hormones

The so-called plant hormones are not directly analogous to animal hormones and are often referred to simply as plant growth substances. They are not necessarily conducted via the vessels of the plant and their molecular form does not subscribe to either category of animal hormone (gibberellins do, however, have a molecular similarity to steroids). Moreover, it has not proved easy to develop assays for plant hormones which differentiate with complete clarity between one plant hormone and another. Some of these hormones are very widely distributed in all plant tissues.

There are three important types of plant growth substances, auxins, gibberellins and cytokinins, with the gas ethylene as a marginal fourth. Auxin has now been identified as indole-acetic acid (IAA) and a wide range of synthetic auxins are now manufactured and employed as herbicides. Effects of auxins include root formation, stem elongation, inhibition of leaf abcission and lateral bud development. At a cellular level, auxin induces cell enlargement in many plant tissues and cell division in some. There is no evidence that auxin actually enters the cell. Instead, it appears to act in a manner analogous to the animal polypeptide hormones. However, the second messenger system involved in auxin activity is not known.

Gibberellins are structurally similar to steroids and possess a gibbane molecular skeleton. At least twenty different natural gibberellins are known, each with slightly variant physiological properties and most plants possess several different kinds. As with steroid hormones, gibberellin probably acts directly on the genetic material of the target cell. Indeed, it is interesting to note that the insect steroid hormone ecdysone affects plant growth (Carlisle *et al.*, 1964) and gibberellin has some activity on the moulting of locusts (Carlisle *et al.*, 1964).

Cytokinins are dehydrated deoxyadenosine molecules and much of the credit for understanding their structure and activity goes to Professor Skoog at the University of Wisconsin. The effects of these substances are rather diverse and all promote cytokinesis or cell division (thus the name cytokinin) but they appear also to have some rather specific differentiating influence on cultures of plant callus tissue (Skoog and Miller, 1957). Cytokinin action involves incorporation of the molecule into cellular RNA, especially transfer RNA, and there are grounds for thinking that they may be incorporated into the transfer RNA molecule next to the anticodon site (Fox and Chen, 1968; reviewed in Steward and Krikorian, 1971). Perhaps their differentiating effects are based on alterations in the population of active tRNA molecules, as discussed in Section 6B.

With a remarkably simple structure $CH_2{=}CH_2$, ethylene is certainly the simplest specific biological activator known. Its effects on plants are chiefly in the area of fruit ripening, leaf abscission and flowering and there is evidence that its main effect lies in altering cell permeability (Burg, 1962).

Plant growth substances (plant hormones) can, therefore, be seen as having some characteristics of conventional animal hormones and in some cases they are transported from a site of synthesis to a target cell elsewhere. But often they are perhaps more analogous to chalones, which modify growth and cell division in the tissue of their origin. The expression of the genes is often greatly modified by these substances but, as with animal hormones, at a variety of levels and in very diverse ways.

3. Chalones

Chalones are tissue specific mitotic inhibitors and their general importance in the regulation of growth and cell division remains problematical. Much of the work on chalones has been carried out by Bullough and his publications argue strongly for their importance (Bullough, 1967). The original experiment of Bucher in 1951 (Bucher *et al.*, 1951), in which two rats had their blood circulation joined in parabiosis and one of the two had part of the liver excised, remains as an important proof of the existence of chalones. In this experiment, the liver of the non-hepatectomized animal displayed hyperplasia. However, although the experiment has often been repeated, isolation and purification of the humoral agent responsible for the effect has proved extremely difficult. Such purification as has been accomplished yields a glycoprotein of low molecular weight. The theory of chalone activity normally advanced is that the cells of a tissue organ will constantly release chalone molecules which, by acting as mitotic inhibitors, prevent growth of that tissue. The molecules are thought to be very unstable, and so, if part of the tissue or organ is removed, the level of circulating specific chalone will rapidly drop, permitting increased cell division in that tissue. Presumably, the inherent instability of chalones explains the difficulties attending their isolation. Any mechanism of chalone action remains theoretical, and their involvement in gene expression and differentiation unknown. Evidence exists suggesting their implication in growth of kidney and epidermal tissues, as well as liver (Bullough, 1967).

4. Phytohaemagluttinin

Phytohaemagglutinin is a glycoprotein complex extracted from kidney beans, capable of agglutinating human red cells of group O. It was also found to have mitogenic (mitosis stimulation) properties on human leucocytes (Carstairs, 1961) and it later became clear that such leucocytes were derived from the small lymphocytes. Pearmain *et al.* (1963) have demonstrated a similar reaction to tuberculin by lymphocytes of persons with a positive tuberculin reaction. However, this induction of mitosis appears to be specific to lymphoid cells and may well be analogous to effects of other antigens or such cells. In any event, the application of such mitogenic phenomena to other cell systems is unlikely and any influence on generalized gene expression minimal.

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Author Index

A

Abbott. J., 153, 156, 157, *302, 307*
Abelson. J. N., 257. *320*
Ada, G. L., 39, *309*
Adams, G. H. M., 167, *292*
Adelberg, E. A., 282, *322*
Adesnik, M., 171, 172, *289*
Ahmed, S. I., 134, *299*
Akhtar, M., 98, *309*
Allerdice, P. W., 145, *320*
Allfrey, V. G., 167, 177, 179, 269, 273, 275, *304, 312, 316, 321*
Allison, W. S., 48, *319*
Aloni, Y., *289*
Alton, T., 260, 262, 277, *306*
Amaldi, F., 254, *289*
Amenson, C. S., 260, 271, *295*
Ames B. N., 166, *289*
Ames, B. W., *292*
Anderson, C. R., 84, *320*
Ansari, A. Q., 98, *309*
Ansevin, A. T., 181, *314*
Apel, K., 214, *289*
Apear, J., 257, *301*
Appella, E., 42, *309*
Arias, F., 99, *320*
Arms, K., 141, *320*
Armstrong, J. J., 248, *315*
Arnott, H. J., 242, *298*
Aronson, A. I., 27, 258, *289*
Arst, H. N., 133, *289*
Aschaffenburg, R., 88, *304*
Askonas, B. A., 38, 83, *289, 290*
Ashworth, J. M., 194, 195, 196, 256, *289, 320*
Attardi, B., 65, 66, 57, 244, 258, *289*
Attardi, G., 65, 66, 67, 244, 253, 257, 258, *289, 301*
Aub, J. C., 288, *292*
Austin, C. M., 39, *309*
Avers, J., 244, 245, *301*
Axel, R., 182, 274, *289*
Axelrad, A. A., 63, *290*

B

Baglioni, C., 59, 74, 172, 263, 270, *290, 291, 303*
Baker, C. M. A., 55, 56, *307*
Baker, W. W., 121, *308*
Baldwin, J. P., 170, 268, 273, *290*
Baldwin, R. L., 84, *322*
Ball, W. D., 95, 105, 106, 107, 108, 154, *312*
Baltimore, D., 21, 67, *290, 317*
Baltus, E., 212, *290, 317*
Banerjee, A. K., 171. *315*
Banerjee, S., 205, 283, *319*
Banks, J., 264, *316*
Barclay, P., 166, *297*
Barker, J. E., 62, *290*
Barlow, P. W., 187, *290*
Barrett, T., 182, 274, *290*
Barry, J. M., 83, *290*
Bartley, J. A., 172, *290*
Barton, S. C., 186, 188, *298*
Basch, J. J., *321*
Baserga, R., 274, *312*
Bateman, A. E., 63, 64, 70, *290*
Batistoni, F. A., 254, *311*
Baulieu, E. E., 99, *322*
Bautz, E. K. F., 26, 27, 174, *290, 293*
Bautz, F. A., 174, *290*

Baynes, Y. A., 72, 143, 175, *321*
Beale, G. H., 242, *290*
Beccari, E., 254, *311*
Becker, A. J., 61, *290*
Becker, H. J., 269, *290*
Beckwith, J., 18, *302*
Beisson, J., 204, *290*
Bektor, I., 181, *290*
Bell, 79, 281, *291, 321*
Benbassat, J., 58, *291*
Benzer, S., 16, *291*
Berendes, H. D., 286, *291*
Berrill, N. J., 207, *291*
Betz, T. W., 55, *307*
Bick, M. D., *295*
Birnstiel, M. L., 23, 38, 171, 172, 255, 257, 270, 271, *291, 304, 306*
Bishop, J. O., 74, 261, 270, *291, 293*
Blacker, A. N., 242, *295*
Blanco, A., 43, *319*
Blobel, G., 69, 263, *291, 297*
Bloom, W., 61, 83, *291*
Blum, N., 70, *291*
Bobrow, S. N., 213, *291*
Bock, R. M., 270, *313*
Bodman, W. S., 193, *301, 320*
Bonner, J., 169, 176, 177, 181, 268, *290, 291, 296, 302, 312, 314, 319*
Bonner, J. T., 197, *291*
Borun, T. W., 172, *291, 303, 312*
Boseley, P. G., 170, 268, 273, *290*
Bourgeois, S., 18, *311*
Boxter, J. D., 163, *316*
Boyd, J. B., *296*
Boyer, H. W., 247, *291*
Boyer, S. H., 88, *320*
Brachet, J., 149, 213, 281, *291*
Bradbury, E. M., 170, 268, 273, *290*
Bradshaw, W. S., 95, 105, 106, 107, 108, 154, *312*
Bramwell, M. E., 72, *301*
Brasch, K., 167, *292*
Braun, A. C., 234, 235, 236, *292*
Brecher, P. I., 286, *303*
Breindl, M., 172, *292*
Brenner, M., *292*
Brenner, S., 16, 261, *292, 295*
Briggs, R., 137, 145, *292*
Brink, R. A., 236, *292*
Britt, O. K., 237, *315*
Britten, R. J., 260, 271, 273, 274, 277, 279, *292, 295*
Brooks, G. T., 61, *306*
Brooks, R. R., 131, *320*
Brostoff, J., 40, *312*
Browlee, G. G., 262, *311*
Brown, D. D., 90, 91, 92, 213, 253, 254, 257, *292, 300, 306, 315, 316*
Brown, J. L., 55, *292*
Brown, S. W., 4, *292*
Bruce, S. A., 155, *315*
Bruns, G. A. P., 58, *292*
Bryant, P. J., 222, 224, 226, 232, *292, 310*
Bucher, M. L. R., 288, *292*
Bull, A. L., 225, *292*
Bullough, W. S., 288, *320*
Buongiorno-Nardelli, M., 254, *289*
Burg, S. P., 287, *293*
Burgess, R. R., 26, 27, 174, *293, 319*
Burgos, H., 70, *310*
Burnet, F. M., 33, *320*
Burnett, A. L., 216, 217, 218, 219, *293, 296*
Burnham, B. F., 64, *293*
Burr, H. E., 68, *306*
Butcher, R. W., 284, 285, *293*
Butterworth, P. A. W., 175, *294*
Byers, T. J., 141, *320*

C

Callan, H. G., 37, 74, 271, *293*
Cameron, I. L., 143, *293*
Campbell, J. C., 78, 80, 81, *293, 294*
Campbell, P. N., 83, *289*
Campbell, R. D., 217, *293*
Campo, M. S., 261, *293*
Caplan, A. I., 155, *293*
Capp, G. L., 55, *293*
Carey, N. H., 102, *295*
Carlisle, D. B., 287, *293*
Carlton, B. C., 16, *319*
Carstairs, K., 288, *293*
Carsten, M. E., 124, *293*
Carter, B. L. A., 112, 115, 116, 280, *300*
Case, M. E., 134, *299*
Castorph, H., 135, *305*
Cedar, H., 182, 274, *289*
Chalkley, R., 172, *290*
Chamberlin, M., 22, *293*
Chambers, D. A., 18, *319*
Champon, P., 175, *304*
Chan, L. L., 9, 68, *294*
Chan, L. N., 226, *293*
Chanduri, S., 274, *322*
Chapelle, A., 62, *296*
Chen, C., *297*
Chesterton, C. J., 175, *294*
Chipchase, M., 23, 255, 271, *291*
Chui, D., 69, *296*
Clark, A. J., 21, *306*
Clark, W. R., 95, 104, 105, 106, 107, 108, 109, 154, *310, 312, 320*
Clauss, H., 212, *294*
Claycomb, W. C., 122, *299*
Clayton, R. M., 75, 76, 77, 78, 79, 80, *294, 318*
Clemens, M. J., 98, 99, *294*
Clutterbuck, A. J., 131, *294*
Cohen, P. P., 164, *294*
Cohn, M., 18, *311*
Cole, R. D., 131, 167, *302*
Cole, R. J., 61, 64, 70, *290, 294*
Coleman, A. W., 121, 156, *294, 320*
Coleman, J. R., 121, 156, *294, 320*
Coleman, R. D., 126, *319*
Comi, P., 65, *299*
Comings, D. E., 167, 168, 171, 176, 260, 271, *294*
Commandon, J., 137, *294*
Conkie, D., 70, *310*
Constock, J. P., 286, *309*
Cook, P. R., 37, 72, 143, *294, 301*
Corbin, J. D., 284, *294*
Cordova, C. C., 146, *309*
Core, D. J., 133, *289*
Corliss, J. O., 203, *294*
Costello, L. A., 50, *294*
Cousineau, L., 46, 48, *315*
Cove, D. J., 131, 132, 133, 135, *294, 295, 310*
Cox, C. G., 116, *295*
Cox, R. F., 9, 101, 102, 103, *295*
Craig, N., 171, *295*
Creanor, J., 113, 115, 117, *297, 308*
Creighton, M. O., 81, *295*
Crick, F. H. C. , 16, 260, 277, *295*
Crippa, M., 174, 255, 257, *295, 316*
Croft, J. H., 245, *314*
Cryer, D. R., 245, *299*
Cunningham, A. J., 38, *295*

D

Dahlberg, D., 280, *319*
Daley, K., 26, *305*
Damsanov, I., 189, *295*
Darlington, A. J., 133, *313*
Darnell, J. E., 39, 171, 172, *289, 313*
Darnell, J. R., 258, *314*
Davidson, E. H., 260, 271, 273, 274, 277, 279, *292, 295*
Davidson, R. I., *296*
Davie, J. M., 38, *320*
Davies, D. R., 126, *304*
Davies, L. E., 217, *295*
Dawid, I. B., 213, 242, 282, *292, 295*
Dawson, D. M., 125, *295*
Dawson, G. W. P., 40, *314*
Delange, R. J., 170, *295*
Denamur, R., 87, *298*
Denis, H., 254, 256, *307*
Desalu, O., 65, *306*
De Sombre, E. R., 286, *303*
De Terra, N., 141, *295*
Dethlefson, L., 163, *316*
Dice, J. F., 13, 51, *299*
Dickerson, R. E., 126, *304*
Diehl, F., 219, *296*
Diggelman, H., 66, 259, *302*
Dill, B. C., 131, 167, *305*
Dippell, R. V., 203, 204, *296, 314*
Disbrey, C., 205, *311*
Dixon, G. H., 167, *303*
Djaldetti, M., 69, 70, 71, *296*
Dominquey, J. L., 270, *313*
Donachie, W. D., 22, 115, *296*
Dorfman, A., 152, 153, 154, 155, 156, 157, *305, 322*
Doudoroff, M., 282, *322*
Drapeau, G. R., 16, *319*
Drewienkiewicz, C. E., 66, 258, 259, *318, 319*
Dublin, D. T., 166, *289*
Dunn, J. J., 26, 27, 174, *293*
Du Praw, E. J., 268, *296*

E

Eason, R., 67, *319*

Echols, H., 19, *298*
Edelman, G., 34, 38, 39, *298*
Edgell, M. M., 242, *302*
Edmonds, M., 10, 260, *309*
Elgin, S. C. R., 176, *296*
Ellis, P. E., 287, *293*
Ellis, R. J., 242, 246, *296*
Ellison, M. L., 154, *296*
Elsdale, T. R., 138, *296*
Elston, R. C., 136, *320*
Emtage, J. S., 9, 101, 102, 103, *295*
Engelmann, F., 266, *296*
Englesberg, E., 19, 20, 278, *296*
Ensign, J. C., 133, *301*
Ephrussi, B., 189, 244, 246, *296, 303*
Eppenberger, H. M., 125, *295*
Equchi, L., 76, 81, *309*
Evans, M., 191, *296*
Everse, J., 46, 51, *296*
Everett, G. A., 257, *301*

F

Failly, C., 191, *303*
Fam, H., 67, *317*
Fanster, B., 173, *296*
Fantoni, A., 62, *296*
Farrell, H. M., 87, *319*
Farusawa, M., 73, *297*
Faulkner, R., 273, *320*
Fawcett, D. W., 61, 83, *291*
Felsenfeld, G., 182, 274, *289*
Feughelman, M., 166, *297*
Ficq, A., 281, *291*
Fincham, J. R. S., 240, *297*
Finck, H., 123, *297*
Fink, G. R., 135, *297*
Firtel, R., 260, 262, 271, 277, *297, 306*
Fischberg, M., 138, *296*
Fischer, S., 70, 280, *297*
Fischer, E. H., 126, *305*
Fitzgerald, P. H., 288, *310*
Flamm, W. G., 269, *297*
Flavell, R. A., 283, *297*
Foguelman, A. I., 132, *313*
Fonbrune, P. de, 137, *294*
Forchhammer, J., 265, *297*
Ford, P. J., 254, *298*
Fordham, S. A., 38, *295*
Forssman, W. G., 107, *298*
Fox, J. E., *297*
Francis, D., 196, 197, *297*
Fraser, M. J., 84, *297*
Fraser, R. C., 62, *297*
Fraser, R. S. S., 117, *297*
Freese, E., *297*
Freienstein, C., 69, *297*
Frenster, J. H., 268, 274, *298*
Friend, C., 60, 73, *298*
Fritz, P. J., 52, *298*
Fuhr, J., 70, 280, *297*

G

Gage, L. P., 89, 90, 91, *298, 316*
Gale, E. F., 7, *298*
Gall, J. G., 255, 271, *298*
Gallwitz, D., 172, *292*
Gallo, R. C., 213, *291*
Gally, J. A., 34, 38, 39, *298*
Garcia-Bellido, A., 232, *298*
Gardner, R. L., 186, 187, 188, *298, 301*
Garen, A., 19, *298*
Garnier, J., 88, *300*
Garrod, D., 195, 196, *320*
Gault, E., 242, *298*
Gaze, P., 87, *298*
Gehring, W., 226, 228, 229, 231, *293, 298, 299*
Gelhrter, R. D., 113, 162, 163, *316*
Georgiev, G. P., 258, 263, 273, 274, 277, *299, 306*
Gershon, R. K., 40, *299*
Gianni, A. M., 65, *299*
Gielen, J., 9, 69, 73, 74, *312*
Gierer, A., 24, 278, *299*
Giglioni, B., 65, *299*
Gilbert, J. B., 116, *295*
Gilbert, W., 18, 24, 177, 261, *299, 300*
Giles, N. H., 134, *299*
Gilmour, R. S., 181, *299, 310*
Glassman, E., 136, *320*
Godin, C., 83, *289*
Goldberg, A. L., 13, 15, *299*
Goldring, E. S., 245, *299*
Goldschmidt, R., 229, *299*
Goldstein, L., 137, 141, *299, 320*
Goldstein, M. A., 122, *299*
Goodenough, V. W., 248, *315*
Goodfriend, T. H., 50, *299*

Goodman, H. M., 257, *320*
Gordon, A. S., 70, *299*
Gordon, W. G., 83, 84, *299, 303*
Gorini, L., 26, *303*
Gould, H. J., 182, 274, *290*
Grace, D. H., 72, *301*
Graham, C. F., 184, 186, 187, *290*
Graham-Smith, R., 213, *291*
Granboulan, N., 259, 263, *300, 314*
Granner, D., 113, 162, 163, *316*
Grant, W. D., 198, *300*
Grasso, J. A., 129, *306*
Greaves, M. F., 40, *312*
Green, M. M., 40, *300*
Greenaway, P. J., 167, *300*
Greenberg, J. R., 259, *300*
Greenberg, R., 83, 84, *299, 303*
Greengard, O., 158, 159, 160, 161, *300*
Gregg, J. H., 193, *320*
Gregory, C. J., 63, *300*
Grimley, P. M., 101, *304*
Grobstein, C., 86, *300*
Gros, F., 261, *300*
Gröschel-Stewart, U., 123, *300*
Grosclaude, F., 88, *300*
Gross, J., 155, *316*
Grossman, L. I., 245, *299*
Grover, A., 99, *316*
Groves, M. L., 83, *299*
Guest. J. R., 16, *319*
Gurdon, J. B., 3, 11, 65, 68, 136, 138, 139, 140, 141, 146, 147, 148, 149, 150, 250, 252, 253, 256, 264, *292, 300, 308, 320, 321, 322*
Gutfreund, H., 84, *297*
Gutman, G. A., 101, *310*
Guttman, B., 18, *300*

H

Hadorn, E., 231, *300*
Haggard, M. E., 57, *313*
Haines, M. E., 9, 101, 102, 103, *295*
Hallberg, R. L., 257, *300*
Halvorson, H. O., 111, 112, 115, 116, 280, *300, 322*
Hamilton, L., 166, *297*
Hamkalo, B. A., *308*
Hamyln, P. H., 182, 274, *290*
Hammaker, L., 64, *313*
Hammerling, J., 208, 209, 210, *300, 301*
Harris, H., 65, 71, 72, 136, 142, 143, 144, 145, 210, 212, 250, 258, 269, *301, 314, 318, 320, 321*
Harris, J. W., 54, *301*
Hart, R. C., 126, *304*
Hartley, M. R., 242, 246, *296*
Hatlen, L. E., 253, 257, *289, 301*
Hauschka, S. D., 122, 129, *301*
Hayasaka, S., 64, *319, 321*
Heald, C. W., 83, *312*
Heath, D. S., 63, *290*
Heil, A., 27, *301*
Heller, P., 126, *319*
Helmstetter, C. E., 23, *301*
Henry, S. A., 111, *322*
Henshaw, E. C., *301*
Herbert, M. C., 184, 186, 187, 188, *301*
Herrmann, H., 120, 127, 128, *301, 307*
Heywood, S. M., 120, 127, 128, *301*
Hiatt, H., 261, *300*
Hickey, I., 73, *310*
Hildebrandt, A. C., 234, *303, 309*
Hilder, V. A., 71, 175, 176, 182, 260, 269, 272, 278, 281, *301, 307, 321*
Hinilica, L. S., 181, *314*
Hirschberg, R., 133, *301*
Hirvonen, A. P., 251, *319*
Ho, T., 100, *318*
Hoffmann, H. P., 244, 245, *301*
Holder, J. W., 68, *306*
Holl, F. B., 132, *313*
Holland, J. G., 73, *298*
Holley, R. W., 257, *301*
Holliday, R., 275, *301*
Holsten, R. D., 234, *315*
Holtzer, H., 127, 153, 156, 157, 276, *302, 307, 322*
Honjo, T., 4, 6, 38, 254, *302, 305*
Hood, L. E., *296*
Hooper, C., 166, *297*
Horton, B. F., 59, *302*
Hottinguer, H., 246, *296*
Houdeline, L., 87, *298*
Hough, B. R., 260, 271, *295*
Hsiang, M. W., 131, 167, *302*
Haung, P. C., 131, 274, *302, 320*
Huang, R. C., 181, 268, *302, 321*
Hubert, E., 69, 149, 264, *291, 321*

Huey, G., 69, 149, 171, 264, *291, 302, 321*
Huggins, C., 155, *311*
Huisman, T. H. J., 59, *302*
Hunt, J. A., 68, *305*
Hunt, T., 64, 280, *302, 321*
Hunter, A. R., 65, 68, *302, 321*
Hunter, T., 280
Hutchinson, C. A., 242, *302*
Huxley, H. F., *321*

I

Ibel, K., 170, 268, 273, *290*
Iio, T., 164, *321*
Ikawa, Y., 9, 69, 73, 74, *297, 312*
Illmensee, K., 226, *302*
Imaizumi, T., 66, 259, *302, 314*
Imamoto, F., *302*
Ingle, J., 240, *302*
Ingram, V. M., 9, 55, 58, 60, 66, 68, 74, 126, 258, *292, 294, 302, 305, 307, 309*
Ippen, K., 18, *302*
Isensee, H., 43, *319*
Ishida, S., *302*

J

Jackson, E. N., 265, *297*
Jackson, G., 155, *316*
Jacob, F., 261, *292, 302*
Jacobsen, K. B., 66, 257, *303*
Jacobs-Lorena, M., 172, 263, *303*
Jacobson, A., 260, 262, 277, *306*
Jacoby, G. A., 26, *303*
Jahred, D. W., 100, *317, 318*
Jailkhani, B. L., 99, *316*
Jami, J., 191, *303*
Jarvorska, H., 271, *306*
Jenness, R., 83, *303*
Jensen, E. V., 286, *303*
Jergil, B., 167, *303*
Jinks, J. L., 282, *303*
Johnson, E. M., 275, *304*
Johnson, R. T., 141, *311*
Jones, C., 51, *311*
Jones, I. G., 283, *297*
Jones, K. Q., 38, *291*
Jones, K. W., 78, 81, 269, *293, 303*
Jones, L. E., 234, *303*
Jones, R. F., 68, *306*
Jones, R. T., 55, *293*
Jones, S. B., 83, *299*
Jordan, E., 254, *292*
Jorgensen, G. N., 83, *305*
Jurd, R. D., 37, 54, 55, 57, 58, 59, 60, 61, 64, 66, 71, 246, 258, 280, *303, 306, 307*

K

Kafatos, F. C., 89, 94, *303, 321*
Kagen, L. J., 127, 128, *303*
Kahan, B. W., 189, *303*
Kaiser, D., 166, *303*
Kalan, E. B., 83, 84, *299, 303, 321*
Kanbysellis, M. P., 96, *303*
Kang, C. Y., *308*
Kankel, D., 156, *294*
Kano-Sueoka, T., 66, 257, *315*
Kao, F. T., 51, *311*
Kaplan, N. O., 46, 48, 50, 51, 125, *294, 295, 296, 299, 304, 312, 319*
Karn, J., 275, *304*
Kardstadt, M., 26, *305*
Kedes, L. H., 171, 172, 270, *304*
Kedinger, C., 175, *304*
Keller, N., 177, 319
Keller, W., 175, *315*
Kellermayer, R. W., 54, *301*
Kelley, D. E., 171, *295*
Kellgher, J. K., 205, 283, *319*
Kemp, J. D., 95, 105, 106, 107, 108, 154, *312*
Kendrew, J. C., 126, *304*
Kennell, D., 23, *304*
Kent, A. E., 234, *315*
Khorana, H. G., 24, 278, *313*
Kiddy, C. A., 88, *304*
Kiho, Y., 251, *304*
King, J. W. B., 88, *304*
King, R., 225, *304*
King, T. J., 137, *292*
Kirby, D. R. S., 184, *304*
Kivilaakso, E., 63, *304*
Klein, G., 144, 145, *318, 321*
Kleinsmith, L. J., 177, 189, *304*
Knopf, P. M., 251, *318*
Knowland, J. S., 146, 147, 253, *304, 305*
Kodaira, K., 252, *309*

Koga, K., 164, *321*
Kohler, P. O., 101, *304*
Komaromi, L., 263, *306*
Konigsberg, I. R., 121, *304*
Kopp, D. W., 10, 260, *309*
Kornberg, A., 170, 173, 268, *304*
Kornberg, R. D., 170, *304, 321*
Kossman, C. R., 23, 253, *304*
Koyama, H., 276, *304*
Krakow, J. S., 26, *305*
Krebs, E. G., 126, 284, *294, 305*
Krikorian, A. D., 287, *315*
Kroeger, H., 281
Krupnick, D., 245, *299*
Krzyek, R., *305*
Kuhn, L., 135, *305*
Kung, G. M., 181, *290*
Kunkel, H. G., 55, *305*
Kuntzel, H., 175, 242, *305*
Kupchyk, L., 43, *319*
Kurland, C. G., 261, *300*

L

Landy, A., 257, *320*
Lane, C. D., 68, 146, 147, 148, *305, 308, 321, 322*
Lane, D., 247, 248, *312*
Lange, R. D., 70, *305*
Langridge, R., 166, *297*
Lanyon, G., 67, *319*
Lark, K., 23, *305*
Larson, B. L., 83, 84, *305, 320*
Lash, J. W., 154, *296, 305*
Laskey, R. A., 138, *300, 321*
Laurence, E. B., 63, *316*
Lava-Sanchez, P. A., 254, *289*
Laycock, D. G., 68, *305*
Lazarow, A., 108, *309*
Lebleu, B., 69, 264, *321*
Leclercqu, M., 69, 264, *321*
Leder, P., 9, 38, 69, 73, 74, *305, 312*
Lee, J. C., 66, 258, *305*
Lehninger, A. L., 117, 123, *305*
Leighton, T. J., 27, 131, 167, *305*
Letnansky, K., 131, 167, *319*
Levenson, G. E., 153, *321*
Levine, L., 48, *319*
Levine, R. P., 248, *315*
Levinson, B. B., 163, *316*
Levitt, D., 152, 153, 154, 155, 156, 157, *305*
Lewin, B., 20, 26, 66, 172, 173, 250, 253, 254, 257, 259, 261, 263, *305*
Lima-de-Faria, A. 271, *306*
Lindell, T. J., 175, *321*
Linder, S., 128, *303*
Lingrel, J. B., 65, 68, 146, 148, 149, *300, 306, 321,* 322
Littau, V. C., 179, 269, 275, *312*
Littauer, U. Z., 69, 264, *321*
Little, C. C., 189, *315*
Littlefield, J. W., 173, *306*
Lizardi, P., 91, *306*
Lloyd, L., 271, *293*
Lockard, R. E., 68, *306*
Lockwood, D. H., 86, *306, 319*
Lodish, H. F., 65, 252, 260, 262, 271, 277, *297, 306, 321*
Loeb, L. A., 173, *296*
Lofthouse, R., 99, *294*
London, I. M., 64, *302*
Losick, R., 27, *306*
Love, D. S., 129, *306*
Lucas, F., 88, *306*
Luck, D. J. L., 242, *306*
Lukanidin, E. M., 263, *306*
Lycette, R. R., 288, *310*

M

Maas, W., 177, *319*
Maas, W. K., 21, *306*
McCarthy, B. J., 66, *313*
McCleod, D. L., 63, *290*
McClintock, B., 40, 238, 239, *308, 322*
McCullock, E. A., 61, 63, *290, 300, 316*
McDevitt, D. S., 78, *308*
MacGillivray, A. J., 274, 277, *306*
McGovern, A. P., 173, *306*
McGrath, J., 22, *293*
McKnight, G. S., 101, *311*
MacLean, N., 37, 54, 55, 56, 57, 58, 59, 60, 61, 63, 64, 66, 67, 68, 71, 72, 74, 91, 122, 143, 175, 176, 182, 205, 215, 246, 251, 258, 259, 260, 271, 272, 278, 280, 281, *301, 303, 306, 307, 310, 313, 316, 321, 322*
McLellan, W. L., 25, *319*
Madgwick W. J., 66, 67, 68, 72, 143, 215, 259, *307*

Madison, J. T., 257, *301*
Maeda, Y., *302*
Mairy, M., 254, 256, *307*
Maleknia, M., 70, *291*
Mangi, R. J., 73, *307*
Maniatis, G. M., 58, *307*
Manwell, C., 55, 56, *307*, *309*
Mapes, M. O. 234, *315*
Marbaix, G., 68, 69, 146, 148, 264, *308*, *321*
Marchok, A. C., 120, 127, 128, *301*, *307*
Mardiney, M. R., 73, *307*
Margeson, K. B., 173, *306*
Margulis, L., 205, 283, *319*
Markert, C. L., 42, 43, 50, 51, 227, *309*
Marks, P. A., 58, 62, 69, 70, 71, 264, *296*, *307*, *316*
Marmur, J., 245, *299*
Marquise, H., 257, *301*
Marrer, H., 64, *313*
Martin, D. W., 113, 162, 163, *316*
Maryanka, D., 182, 274, *290*
Masters, M., 22, 115, 116, *296*, *307*
Mathews, M. B., 132, 171, 261, *307*
Matioli, G. T., 57, *307*
Matsubaru, M., 253, *307*
Matsumra, S., 89, *316*
Maxam, A., 24, *299*
Mayne, H., 276, *302*
Mayne, R., 156, *307*
Means, A. R., 101, 286, *308*, *309*
Meins, F., 235, *292*
Menza, I., 78, *308*
Meronk, F., 278, *296*
Merrill, S. H., 257, *301*
Meselson, M., 261, *292*
Mester, J., 99, *322*
Metzenberg, R. L., 131, 133, 135, *308*
Meynell, G. G., 28, *308*
Michaelis, G., 245, *308*
Migeon, B. R., 3, *308*
Miki-Noumura, T., 124, *308*
Miller, C. O., 287, *313*
Miller, C. S., 3, *308*
Miller, D. A., 145, *320*
Miller, J. K., 18, *302*
Miller, L., 253, *304*
Miller, O. J., 145, *320*, *322*
Miller, O. L., *308*
Mintz, B., 121, 186, 187, *308*
Mirsky, A. E., *321*
Mitchell, H. K., 244, *308*
Mitchell, M. B., 244, *308*
Mitchison, J. M., 112, 113, 114, 115, 116, 198, *308*
Miura, Y., 276, *308*
Mizutami, S., *308*
Moar, B., 172, *308*, *322*
Moar, V. A., 68, *310*
Mochan, B., 276, *302*
Monod, J., 261, *302*
Moore, G. P. M., 175, *308*
Moorhouse, J. E., 287, *293*
Morata, G., 232, *298*
Morel, C., 263, *314*
Morgan, C. R., 108, *309*
Morimoto, T., 89, *316*
Morris, P. W., 157, 175, 276, *312*, *321*
Morrison, M. 67, *319*
Morrowily, H. J., 23, 253, *312*
Moss, B., 55, *309*
Mueller, K., 174, *309*
Muench, K. H., 257, *309*
Muir, W. H., 234, *309*
Mullen, R. J., 187, *309*
Muller-Hill, B., 18, 177, *299*
Mulnard, J. G., 188, *309*
Mumkres, K. D., 244, *319*
Munday, K. A., 98, *309*
Munro, A., 280, *321*
Murray, K., 167, 181, *300*, *302*

N

Nagata, S., 89, *316*
Nagel, R. L., 70, 280, *297*
Nakazato, H., 10, 260, *309*
Nan, M., 38, *305*
Naora, H., 252, *309*, *318*
Narayanan, P. R., 99, *316*
Nardi, I., 254, *311*
Naresimhan, C., 99, *316*
Needham, D. M., 117, 118, 123, *309*
Neelin, J. M., 167, *292*, *309*
Nemer, M., 172, *322*
Netter, P., 245, 248, *309*
New, D. A. T., 184, *309*
Newbold, J. L., 242, *302*
Newly, R. F., 18, *311*
Nichol, J. M., 100, *318*

Niewisch, H. G., 57, *309*
Niu, L. C., 146, *309*
Niu, M. C., 146, *309*
Nooden, L. D., 177, *317*
Norman, B., 38, *305*
Nossal, G. J. V., 39, *309*
Novick, A., 18, *300*
Novick, R. P., 28, *309*
Nudel, U., 69, 264, *321*
Numata, M., 286, *303*
Nur, U., 4, *292*
Nuret, P., 175, *304*

O

Okada, T. S., 76, 81, *309*
Okazaki, K., 127, *322*
O'Keefe, D. W., 164, *321*
Oldroyd, D., 98, *309*
O'Malley, B. W., 101, 286, *304*, *308*, *309*
Ono, T., 276, *304*
Oosawa, F., 124, *308*
Orci, L., 107, *297*
Osawa, S., 253, *307*
Osborne, D. J., 287, *293*
Otsuki, E., 88, *316*
Ottolenghi, S., 65, *299*
Owen, D. A. J., 187, *290*

P

Packman, S., 9, 38, 69, 73, 74, *305*, *312*
Palleroni, N. S., 133, *310*
Palmiter, R. D., 101, 102, *310*
Papaconstantinou, J., 80, 81, *310*
Papaioamnou, V. E., 186, 188, 232, *298*
Pardee, A. B., 116, *309*
Parnas, H., 65, 66, 67, 258, *289*
Partridge, C. W. H., 134, *299*
Pateman, J. A., 132, 135, *295*, *310*
Paterson, B., 127, 128, *310*
Paul, J., 66, 67, 70, 73, 181, 258, 259, 274, 277, *294*, *299*, *306*, *310*, *319*
Paul, W. E., 38, *320*
Pavlovic-Kentera, V., 70, *305*
Pearmain, C., 288, *310*
Pearson, M. J., 221, *310*
Pearson, P. L., 145, *320*
Pemberton, R., 74, 270, *291*
Penman, M., 262, *310*
Penman, S., 257, 262, *310*, *319*
Penswick, J. R., 257, *301*
Perez, J., 55, *310*
Perrodin, G., 245, *314*
Perry, R. P., 171, 259, *295*, *300*
Pesoe, A., 48, *319*
Peterson, J. A., 72, *310*
Peterson, R. F., 83, *299*
Petrochilo, E., 245, *308*
Pettijohn, D. E., 23, 253, *304*
Pfleiderer, G., 42, 48, *318*
Phillips, C., 131, 167, *305*
Phillips, D. C., 126, *304*
Picket, R. L., 104, 107, 109, 157, 276, *297*, *310*, *312*
Pierce, G. B., 189, *304*, *311*
Pilone, G. B., 254, *311*
Piternick, L., 229, *299*
Platt, D. B., 141, *320*
Playfair, J., 40, *312*
Porter, K. R., 31, 120, *311*
Postlethwait, J. H., 223, 226, 227, 232, *310*
Potter, S. S., 242, *302*
Power, J., 19, *311*
Preer, J. R., 202, *311*
Preisler, H., 70, 71, *296*
Prescott, D. M., 137, 143, *293*, *299*
Pritchard, M., 252, *309*
Proudfoot, N. J., 262, *311*
Pruitt, K. M., 52, *298*
Puck, T. T., 51, *311*
Pugh, J. E., 275, *301*
Puiseux-Dao, S., 206, 209, 210, 212, 214, *311*, *313*
Pujolle, J., 88, *300*
Purdom, I. F., 38, 257, *291*

R

Ramanis, Z., 247, *312*
Randall, J., 205, *311*
Rao, P. N., 141, *311*
Reddi, A. H., 155, *311*
Reeder, R. H., 4, 6, 254, *302*, *311*
Reichlin, M., 48, *321*
Reimann, E. M., 284, *294*
Reitz, M. S., 213, *291*

Remensberger, P., 231, *311*
Renold, A. E., 107, *297*
Revel, M., 69, 264, *321*
Rhoads, R. E., 101, *311*
Ribadeau-Dumas, B., 88, *300*
Rich, A., 251, *304, 318*
Richter, G., 212, *311*
Rifkind, R. A., 58, 62, 69, 70, 71, *296, 307*
Rigas, D. A., 55, *293*
Riggs, A. D., 18, *311*
Riker, A. J., 234, *303, 309*
Ringertz, N. R., 175, *308*
Ripoll, P., 232, *298*
Ris, H., 167, *311*
Risebrough, R. W., 261, *300*
Ritossa, F. M., 253, 255, 257, *311, 314*
Ritz, E., 191, *303*
Robbins, E., 172, *291, 312*
Roberts, C. F., 132, *312*
Robinson, G. A., 284, 285, *293*
Roeder, R. G., 4, 175, *311, 312, 321*
Rogers, P., *305*
Roitt, I. M., 40, *312*
Roman, H., 246, *296*
Ronzio, R. A., 95, 105, 106, 107, 108, *312*
Rosenberg, M., 58, *312*
Rosenfeld, G. C., 286, *309*
Ross, J., 9, 69, 73, 74, *312*
Rouiller, C., 107, *297*
Rubin, M. S., 245, *319*
Rucknagal, D. L., 88, *320*
Rudall, K. M., 88, *306*
Ruiz-Carrillo, A., 179, 269, 275, *312*
Rungger, D., 65, *299*
Russer, G., *312*
Rutter, W. J., 95, 104, 105, 106, 107, 108, 109, 154, 157, 175, 276, *310, 312, 320, 321*
Ruud, J. T., 60, *312*
Ryan, J. L., 23, 253, *312*
Rytomaa, T., 63, *304*

S

Saacke, R. G., 83, *312*
Sadgopal, A., 176, *312*
Safille, P. A., 257, *309*
Sager, R., 246, 247, 248, 281, *312*
Salomon, R., 69, 264, *321*
Salthe, S. N., 50, *312*
Samarina, O. P., 263, *306*
Samuels, H. H., 113, 162, 163, *316*
Sanchez de Jimenez, E., 270, *313*
Sanders, T. G., 95, 105, 106, 107, 108, 154, *312*
Sato, T., 73, *298*
Sauer, H. W., 198, 199, *313*
Scaife, J., 18, *302*
Scazzochio, C., 132, 133, *313*
Schafer, K. P., 175, 242, *305*
Schaffer, B. M., 195, *313*
Schapira, G., 70, *291*
Scharff, M. D., *291*
Scheib, D., 77, *313*
Scher, W., 73, *298*
Scherrer, K., 66, 259, 263, *300, 302, 314*
Schimke, R. T., 13, 51, 101, *311, 313, 322*
Schneider, R. G., 57, *313*
Schneiderman, H. A., 223, 227, 232, *310*
Schoch, H. K., 46, 48, *315*
Scholnick, P., 64, *313*
Schubiger, G., 226, *310*
Schwartz, A., 122, *299*
Schweiger, H. G., 211, 214, *289, 322*
Schweizer, E., 135, *305*
Scott, J. F., 288, *292*
Scott, R. B., 79, *322*
Searls, R., 152, 155, *313*
Sedat, J. W., 22, *313*
Seeds, W., 166, *297*
Seifart, K. H., 174, *313*
Sekiya, T., 24, 278, *313*
Sells, B. H., 257, *291*
Serall, J. S., 177, *317*
Shaw, A. R. E., 56, 58, *313*
Shearer, R. W., 66, *313*
Shephard, D., 208, 214, *313*
Sheppard, D., 20, 278, *296*
Shieness, O., 69, *313*
Shimuyi, H., 84, *297*
Shore, V. C., 126, *304*
Shreeve, M. M., 63, *290*
Sidebottom, E., 72, *301*
Sierra, M. F., 245, *317*
Sigel, R. B., 22, *315*

Silagi, S., 155, *313*
Singh Klar, A. J., 111, *322*
Six, E., 209, *313*
Skoog, F., 287, *313*
Sloninski, P. P., 245, 248, *308, 309, 314*
Smart, J. E., 169, *314*
Smith, E. L., 170, *295*
Smith, J. D., 257, *320*
Smith, L. D., 145, *314*
Smith, P., 131, 167, *319*
Smith, S., 138, *296*
Smith-Keary, P. F., 40, *314*
Sobell, H. M., 24, 278, *314, 322*
Soeiro, R., 258, *314*
Sokol, D. M., 50, *299*
Solter, D., 189, 190, *295, 314*
Sonenshein, A. C., 27, *306*
Sonneborn, T. M., 200, 201, 202, 204, *290, 314*
Sopori, M. L., 99, *316*
Soreqi, H., 69, 264, *321*
Southern, E. M., 254, *297*
Spadari, S., 253, *314*
Spelsberg, T. C., 181, *314*
Spencer, T., 212, *314*
Spiegelman, S., 253, *311*
Spiers, J., 23, 38, 255, 271, *291*
Spirin, A. S., 11, 264, *314*
Sphor, G., 259, 263, *314*
Squires, C., 278, *296*
Stamato, D., 23, 253, *304*
Stamatoyannopoulos, G., 75, *314*
Stanier, R. Y., 133, 282, *310*
Starkweather, W. H., 46, 48, *315*
Steens-Lievens, A., 212, *317*
Steeves, T. A., 240, *315*
Steffensen, D. M., 253, *319*
Stein, G., 274, *322*
Stellwagen, R. H., 276, *315*
Stern, M. S., 186, *315*
Stevens, A., 174, *315*
Stevens, L., 174, 189, *315*
Stevens, L. C., 189, *315*
Stevenson, G. T., 31, 32, 34, *315*
Steward, F. C., 234, 287, *315*
Stock, J. J., 131, 167, *305*
Stockdale, F. E., 86, *315*
Stoddard, F. J., 129, *306*
Stokes, A., 166, *297*
Stolzfus, C. M., 171, *315*
Stoutemeyer, V. T., 237, *315*
Strandberg, B. E., 126, *304*
Strates, B. S., 155, *317*
Street, H. E., 240, *315*
Strokman, R. C., 127, 128, *310*
Sueoka, N., 66, 257, *315*
Sugano, H., 73, *297*
Sugden, B., 175, *315*
Summers, W. C., 22, *315*
Sung, M., 167, *303*
Surzychi, S. J., 246, 248, *315*
Sussex, I. M., 240, *315*
Sutherland, E. W., 284, 285, *293*
Suzuki, E., 90, 91, *316*
Suzuki, Y., 90, 91, 92, *315, 316*
Svenberg, A., 39, *309*
Swan, D., 38, *305*

T

Tabor, C. W., 166, *303*
Tabor, H., 166, *303*
Takata, R., 253, *307*
Takeichi, M., 76, 81, *309*
Takeuchi, I., 194, 195, *302, 316*
Talwar, G. P., 99, *316*
Tamaoki, T., 164, *321*
Tani, S., *302*
Tartar, V., 202, *316*
Tashiro, Y., 88, 89, *316*
Tata, J. R., 99, 164, *294, 316*
Tauro, P., 112, 115, 116, 280, *300*
Temin, H. M., *308*
Temple, G. F., 67, *317*
Teng, C. S., 177, *316*
Teng, C. T., 177, *316*
Terada, M., 264, *316*
Tewari, K. K., 248, *316*
Thomas, C. A., 278, *321*
Thomas, D. Y., 244, *322*
Thomas, J. O., 170, *304*
Thomas, N., 63, 122, *316, 321, 322*
Thompson, E. B., 113, 162, 163, *316*
Thompson, M. P., 87, 88, *304, 317*
Thornley, A. L., 63, *316*
Thorp, F. K., 154, *322*
Threlfall, G., 274, 277, *306*
Till, J. E., 61, 63, *290, 300, 316*
Tingle, M., 111, *322*

Tochini-Valentini, G. P., 174, 255, *293, 316*
Tomkins, G. M., 113, 162, 163, 276, 315, *316*
Toole, B. P., 155, *316*
Topper, Y. J., 86, *306, 315, 317*
Torrigiani, G., 40, *312*
Townsend, R. E., 83, *299*
Travers, A. A., 26, 27, 174, *293, 317*
Trevithick, J. R., 81, *295*
Triplett, E. L., 212, *317*
Truman, D. E. S., 76, 80, 158, 161, 163, *294, 317, 318*
Tsaner, R., *312*
Tuboi, S., 64, *319*
Tuchman, J., 260, 262, 277, *306*
Turkington, R. W., 84, 85, 86, *306, 319*
Tzagaloff, A., 245, *319*

U

Urist, M. R., 155, *317*
Urm, E., 177, *319*
Ursprung, H., 43, 50, 51, 227, *307*

V

Van den Brock, H. W. J., 177, *317*
Vanderhoff, G., 64, *302*
Vaughan, M. H., 258, *314*
Venkatesen, S., 99, *316*
Verma, I. M., 67, *317*
Vesco, C., 262, *310*
Vessell, E. S., 52, *298*
Vidali, G., 275, *304*
Vogel, H. J., 25, 26, *317*
Vogel, R. H., 25, 26, *319*

W

Wake, R. G., 84, *322*
Waley, S. G., 78, *317*
Walker, M., 84, *303*
Walker, P. M. B., 269, *317*
Wallace, R. A., 98, 100, *317, 318*
Wallenius, C., 55, *305*
Walsh, D. A., 284, *294*
Warburton, D., 145, *320*
Ward, C., 280, *319*
Wareing, P. F., 236, 237, *318*
Warner, J. R., 251, 258, *318*
Warren, J. C., 99, *320*
Waskell, L., 22, *293*
Watson, J. D., 261, *300*
Watson-Williams, E. J., 88, *320*
Watts, J. W., *301*
Waugh, L. J., 179, 269, 275, *312*
Weatherall, D. J., 88, *320*
Webb, F. H., 270, *313*
Webster, G., 217, *318*
Weinberg, F., 175, *321*
Weiner, F., 144, *318*
Weintroub, H., 276, *302*
Weiss, M. C., 72, *310*
Wensink, P. C., 254, *292*
Werner, I., 156, *294*
Werz, G., 209, *318*
Wessels, N. K., 106, *318*
White, E. L., 52, *298*
White, P. R., 236, *318*
Whitehouse, H. L. K., 136, *318*
Whitelam, J. M., *318*
Whitten, W. K., 187, *309*
Weidmann, M., 9, 68, *294*
Weiland, T., 42, 48, *318*
Wigzell, H., 34, *318*
Wildman, S. G., 248, *316*
Wilkie, D., 244, *322*
Wilkins, M. H. F., 166, *297*
Wilkinson, J. H., 43, 44, 47, *318*
Wille, J. J., 204, *318*
Williams, C. M., 89, 95, 96, *303*
Williams, R. H., 104, 107, 109, *310, 322*
Williamson, A. R., 38, *289*
Williamson, R., 66, 67, 80, 258, 259, 261, *318, 319*
Wilson, A. C., 48, *319*
Wilson, D. A., 278, *319*
Wilson, H., 166, *297*
Wilson, I. B., 186, *315*
Wilt, F. H., 106, 276, *308, 318*
Wimber, D. E., 253, *319*
Winston, M., 205, 283, *319*
Wintersberger, V., 131, 167, *319*
Withier, P., 51, *311*
Withycombe, W. A., 43, *318*
Wolfson, R., 126, *319*
Wolpert, L., 195, 232, *319*
Woodland, H. R., 3, 65, 68, 140, 141, 146, 148, 149, *300, 321, 322*

Woodward, D. O., 244, *319*
Work, T. S., 83, *289,*
Wray, W., *296*
Wright, B. E. G., 38, 280, *289, 319*
Wu, F. C., *296*
Wu, J. H., 234, *303*

Y

Yaffe, D., 120, 121, 128, *319*
Yakulis, V., 126, *319*
Yamada, T., 78, *308*
Yamagishi, T., 27, *319*
Yanf, H., 177, *319*
Yanofsky, C., 16, 265, *297, 319*
Younger, K. B., 205, 283, *319*

Z

Zadrazil, S., 257, *320*
Zalmanzov, E. S., 263, *306*
Zamir, A., 257, *301*
Zarafonetis, C. J., 46, 48, *315*
Zetsche, K., 213, 214, *319*
Zillig, W., 26, *301*
Zinkham, W. H., 43, *319*
Zubay, G., 18, 177, *317, 319*
Zylber, E. A., 257, *319*

Subject Index

A

- *Acetabularia*
  - anucleate, cap formation, 212
    - RNA synthesis in, 213
  - biochemical aspects, 210–214
  - cap formation, 210, 211
  - chloroplasts in, autonomy, 214
  - differential gene function and, 205–215
  - grafting proceedure in, 208, 209
  - life history, 206–208
  - mRNA in cytoplasm, 264
  - nuclear transplants, 209, 210
  - protein synthesis, 211–213
- *Acheta,* gene amplification in, 271
- Acinar cells in exocrine pancreas, 105
- *Acrasieae,* differential gene function and, 192
- Actin
  - in muscle cells, 123, 124
  - in muscles, control of level, 128
- Actinin in muscle cells, 122, 123, 125
- Actinomycin D
  - crystallin synthesis and, 79, 80
  - inducible enzyme synthesis in liver, 162, 163
  - transcription and, 7
- Adenosine monophosphate
  - cyclic, cellular slime moulds cell aggregation and, 192
    - hormone actions mediated by, 284, 285
- Adrenal glands, LDH subunits in, 46, 47
- Adrenocorticotropic hormone actions mediated by cyclic AMP, 285
- *Agrobacterium tumefaciens,* crown gall in plants and, 234
- Alanine tRNA, sequence, 257
- Aldolase synthesis in anucleated *Acetabularia,* 211, 212
- Allelic exclusion, immunoglobulin genes, 36, 37
- Allotypic identity in immunoglobulin genetics, idiotypic variation and, 37, 38
- Allotypic variation immunoglobulin, 32
- α-Amanatin, transcription and, 8
- *Ambystoma mexicanum*
  - genetic defects in, microinjection of cell contents and, 145
  - haemoglobins in, 55
- Amino acids availability, gene expression and, 12
- Amphibia
  - liver, enzyme synthesis in, 163, 164
  - nuclear transfer in, by micromanipulation, 137
  - red blood cells in, 60
- Amplification of genes, 271, 272
- Amylase
  - manufacture, in exocrine pancreas, 105
    - in pancreas, differentiation and, 107
  - in pancreas, 104
- Anaemia, erythropoietin and, 71
- Animal hormones, gene expression and, 283–286
- Antibiotics, transcription and, 7
- Antibodies
  - diversity, 33
  - production, 30
    - cellular control, 40, 41
- *Antirrhinum,* unusual genetic elements in, 238–241
- L-Arabinose operon in *Escherichia coli,* 19
- *Aspergillus*
  - differential gene function in, 130–136
  - enzyme induction by product, 133
- *Aspergillus nidulans,* xanthine dehydrogenase II, negative control, 132
- *Antennapedia,* development commitment in, 227
- ATPase, differential gene function in eukaryotes and, 176–181
- Auxins, gene expression and, 286
- Avidin synthesis, 100

B

- *Bacillus subtilis*
  - enzyme synthesis in, 116
  - multiple copies of single cistrons in, 23
- Bacteria,
  - extrachromosomal genetic factors in, 27, 28
  - genes for RNA in, 253
  - gene nature in, 16
  - genetic organization in, 15
  - LDH in, 51
  - operons, 17–19
  - post-transcriptional controls of gene expression in, 25
  - protein synthesis in, temporal regulation of, 22
  - RNA polymerase, post-transcriptional control of, 26
  - transcription, mechanism, 23
- Bacteriophages, translocon hypothesis and, 40
- Bacteriophage λ, genetic organization in, 15
- Bacteriophage T$_2$
  - genetic organization in, 15
  - mutable site in, 16
- Barr bodies, 17
- Base sequences, recognition by tertiary structure, 24
- Basophilic erythroblast, 61
- B cells, 40
- Birds
  - haemoglobins in, 55
  - LDH$_c$ in, 44
  - red blood cells in, 60
- Brain, LDH subunits in, 46, 47

C

- Calf thymus
  - chromatin, composition, 176
  - histone from, characteristics, 167
- Carbonyl phosphate synthetase I in amphibian liver, 164
- Carrots from isolated phloem parenchyma cells, 234
- Cartilage
  - differential gene function in eukaryotes and, 151–158
  - differentiation, 5-bromo-2′-deoxyuridine and, 155
    - in organ transplants, 155
    - *in vitro,* 153, 154
    - *in vivo,* 155
- Casein metabolism, 83, 84
- α-Casein synthesis, 83
- β-Casein synthesis, 83
- Catecholamines, actions mediated by cyclic AMP, 285
- *Cecidomyidae*
  - gene elimination in, 3
- Cell contents, microinjection, 145, 146
- Cell fusion, differential gene function in eukaryotes and, 141–145
- Centrioles, DNA, 281
- Chalones, gene expression and, 287, 288
- Chickens
  - egg yolk protein synthesis in, 98
  - erythrocytes, high molecular weight in, 66
  - haemoglobins in, ontogeny and, 55
- *Chlamydomonas,* chloroplast genetics in, 246–248
- Chloroplasts
  - in *Acetabularia,* autonomy of, 214
  - in *Chlamydomonas,* genetics, 246–248

  differential gene function in, 241–249
  DNA, 281–283
  genetic and gene expression, 241–244
Chondrocytes
  culture, 153
    5-bromo-2′-deoxyuridine and, 156
  differentiation in culture, 154
Chrondroitin sulphates, 152
Chromatin condensation, 4, 5, 268, 269
    histone and, 169
  eukaryotic, reconstitution, 181, 182
  histone in, structure, 172
Chromosomes
  gene masking and structure of, 266–279
  master-slave models in organization of, 37
Chymotrypsinogen
  in exocrine pancreas, differentiation and, 106
  manufacture in exocrine pancreas, 105
Cilia, basal bodies, DNA, 281
Clonal selection hypothesis for antibody diversity, 33
Clustering of genes for light and heavy immunoglobulin chains, 36
Cocoonase
  experimental systems of differential gene function in eukaryotes and, 93–97
  structure, 94
  zymogen, structure, 94
    synthesis, 94, 95
Col factor, bacterial, 28
Colchicine, milk production and, 86
Coliphage P1, 28
Coliphage T7, multiplication in, 22
Collagen
  effect on myoblasts and myogenesis, 122
  in cartilage, 152
Conalbumin, synthesis, 100
Conifers, phase changes in, 237
Cortical inheritance, Protozoa, 200–205
Creatine kinase
  in muscle cells, 125
  in muscles, control of level, 128, 129
Crown gall in plants, 234–236
Crystallins, 75
  differential distribution, 79
  experimental systems of differential gene function in eukaryotes and, 75–82
  ontogenic succession of, 78
  synthesis, regulation, 76–78
Culture, mammalian embryo, 184
Cyclostomes, haemoglobin in, structure, 54
Cytokinins, gene expression and, 286
Cytoplasm
  mRNA use in, 264, 265
  post-transcriptional control of gene expression and, 279–283

D

Density, variable, genes, 267
Determination in mammalian embryo, 184
Development commitment in Drosophila imaginal discs, 224–226
Dictyostelium
  differential gene function and, 192
  enzyme synthesis in, 195, 196
  life history, 193
Dictyostelium mucoroides, enzyme synthesis in, 197
Differential gene function
  eukaryotes, experimental systems, 30–249
Differentiation
  cartilage, 151
    5-bromo-2′-deoxyuridine and, 155–157
    in vitro, 153, 154
    in vivo, 155
  of cells, tRNA and, 257
  in cellular slime moulds, 194
  chondrocytes in culture, 154
  in *hydra,* 219, 220
  integration of gene expression in, 276–279
  lens fibre, *in vitro,* 81, 82
  in mammalian embryos, 184

  mammary gland, hormonal influence on, 84–86
  muscle, 121
  in organ transplants, 155
  pancreas, 106
    insulin synthesis and, 109
  teratocarcinomas, 190
  yeast cells, 111
DNA
  cytoplasmic, 281–283
  differential gene function in eukaryotes and, 165–183
  packaging in sperm, 166
  in prokaryotes and eukaryotes, 273
  repeated sequences, 269–272
  sequences, transcription, 3, 4
DNA polymerase, differential gene function in eukaryotes and, 172, 173
DNAse, differential gene function in eukaryotes and, 176–181
Drosophila
  eggs, development, 225
  gene clustering in, 136
  gene copies of rRNA in, 255
  genes for RNA, 253
  haploid content, 131
  homeotic mutants, 226–229
  imaginal discs in, differential gene function and, 221-233
    transdetermination in, 275
  phenocopies in, 229
  sex determination by aneuploid situation, 267
  somatic rearrangement of chromosomal material in, 40
  tyrosine tRNA in, 258
Ducks, erythrocytes, high molecular weight RNA in, 66
Dynoflagellates, chromatin, basic protein in, 167
Dytiscus, gene amplification in, 271

E

Ecdysone, zymogen cell differentiation and, 95
Egg proteins, vertebrate, experimental systems of differential gene function in eukaryotes and, 97–103
Egg white proteins
  experimental systems of differential gene function in eukaryotes and, 100–103
  synthesis, regulation by steroids, 101, 102
Egg yolk proteins, production, 98
Elimination of genes, control of expression by, 3
Embryos
  mammalian, differential gene function in, 184–192
  skeletal muscle development in, 120–122
  vertebrate, erythroid cells in, 60
  yolk platelets, DNA, 281
Environment, gene expression and, 202
Enzymes
  in liver, hormonal control, 161, 162
    inducible synthesis in, 162, 163
  synthesis, in amphibian liver, 163, 164
    during yeast cell cycle, 111–117
Erythrocytes, 61
  as terminally differentiated cell, 71
  in fused hybrid cells, 71, 72
  LDH subunits in, 46, 47
Erythroid cells
  haemoglobin in, 59–63
  mammalian, tissue culture, 63
Erythroleukaemic cells, haemoglobin synthesis by, 73
Erythropoietin, haemoglobin synthesis and, 71, 72
Escherichia coli
  alkaline phosphatase in, positive control, 19
  L-arabinose synthesis in, positive control, 19
  arginine biosynthesis in, post-transcriptional control, 25
  genes for RNA, 253
  genetic organization in, 15
  lactose metabolism, operons for, 17
  multiple copies of single cistrons in, 23

- L-rhamnose metabolism, positive control, 19
- RNA polymerase, 174
- rRNA in, 257
- tyrosine tRNA genes in, 257
  - axially symmetrical regions, 24

- Ethylene, gene expression and, 286
- Euchromatin, 267
  - acidic protein in, 176
  - facultative heterochromatin and, 4
- Eukaryotes
  - differential gene function in, experimental systems, 30–299
  - gene regulation in, 266–288
  - ribosomal RNA in, 250
- Experimental systems, 2
  - differential gene function in eukaryotes, 184–249
    - single protein, 30–103
  - post-transcriptional regulation, 13
  - transcriptional regulation and, 7–9

F

- Fibroblasts, effect on myoblasts and myogenesis, 122
- Fibroin
  - experimental systems of differential gene function in eukaryotes and, 88–93
  - mRNA, isolation, 91, 92
- Fish
  - haemoglobins in, 55
  - lactate dehydrogenase in, 43
  - LDH_e in, 44
  - red blood cells in, 60
- α-Foetoprotein in liver, 164, 165
- Frogs, genes for RNA in, 253
- Fungi
  - gene expression in, 130–136
  - LDH in, 51
- Fused hybrid cells, erythrocytes in, 71, 72

G

- Galactokinase in *Aspergillus*, genetic control, 132
- Galactose-1-phosphate uridyl transferase in *Aspergillus*, genetic control, 132
- Gall midges—*see* Cecidomyidae
- Genes
  - amplification, 271, 272
  - clustering, immunoglobulin light and heavy chains, 36
  - controlled transcription of single, 272–276
  - expression, in prokaryotes, 14–29
    - sequential control of, 21–23
    - RNA and, 250–265
  - fungal, clustering, 133–135
    - positive control, 131–133
  - histone, 171, 172
  - inactivated blocks, 267–269
  - masking, chromosome structure and, 266–279
  - multiple copies in prokaryotes, 23
  - nuclear activity, by cytoplasm, 280, 281
  - regulation, general concepts, 266–288
  - for ribosomal RNA, 252–255
    - amplification and magnification of, 255–257
  - sequence, control by other sequences, 5, 6
- Genetic factors, extrachromosomal, in bacteria, 27, 28
- Genetic organization in prokaryotes, 15, 16
- Genomes
  - fungal, 130, 131
    - proteins associated with, 131
  - globin gene frequency in, 73, 74
- Germ line theory for antibody diversity, 33
- Giant polytene chromosomes, chromomeric chromatin in, 271, 272
- Gibberellins, gene expression and, 286
- Globin
  - distribution, 54
  - gene, frequency in genome, 73, 74
  - haemoglobin synthesis control by globin post transcription, 70
  - mRNA haemoglobin synthesis and, 67–69
  - specific mRNA for, 261

- Glucagon
  - actions mediated by cyclic AMP, 285
  - liver enzyme control by, 161
  - manufacture, in endocrine pancreas, 107
    - in pancreas, 104
- Glucose-1-phosphate synthesis in anucleated *Acetabularia*, 211, 212
- Glycans in cartilage, 152
- Grafting, *Acetabularia*, 208, 209

H

- Haem structure, 54
  - synthesis, haemoglobin synthesis and, 64, 65
- Haemocytoblasts, 61
- Haemoglobin
  - cellular distribution, 57
  - distribution, 54-59
  - embryonic, 55, 56
  - experimental systems of differential gene function in eukaryotes and, 53–75
  - foetal, production in adult life, 59
  - (Hb)A, 55
  - (Hb)A_2, 55
  - (Hb)F, 55
  - multiple copies of genes, 270
  - regulation after translation, 280
  - structure, 54
  - synthesis, control, 63–71
- Heart, LDH subunits in, 46, 47
- *Hedera helix*, phase changes in, 236–238
- HeLa cells, chromatin, composition, 176
- Heterochromatin, 267
  - acidic protein in, 176
  - constitutive, 3
  - facultative, 4
- Heterokaryons
  - by cell fusion, 142, 143
  - chicken erythrocytes in, globin synthesis and, 71
- Histone
  - in chromatin, structure, 172
  - differential gene function in eukaryotes and, 166–172
  - DNA and, 273
  - genes, 171, 172
  - mRNA, half life, 171
  - multiple copies of genes, 270
  - specific mRNA for, 261
  - structure and, function, 167–170
- Honeybees, haploid-diploid differentiation in, 266
- Hormones
  - liver enzyme control by, 161, 162
  - mammary gland differentiation and synthesis and, 84–86
  - RNA and gene expression and, 283–288
- Horseshoe crab, LDH in, 51
- Humans
  - haemoglobin distribution in, 55
  - LDH distribution in tissues of, 47
  - sex determination by aneuploid situation, 267
- *Hydra*
  - biology, 216, 217
  - differential gene function in, 215–221
  - explanted tissue, regeneration, 217–219
- Hydrocortisone
  - liver enzyme control by, 161
  - mammary gland differentiation and synthesis and, 85
- Hymenoptera, haploid-diploid differentiation in, 266
- Hypothalamic releasing factors, actions mediated by cyclic AMP, 285

I

- Idiotypic variation
  - immunoglobulin, 32
  - in immunoglobulin genetics, allotypic identity and, 37, 38
- Imaginal discs in *Drosophila*, 221–233
- Immunoglobulins
  - experimental systems of differential gene functions in eukaryotes and, 30–41

  heavy chains, gene clusters for, 34, 36
  light chains, gene clusters for, 34, 36
  structure, 31–33
  synthesis, allellic exclusion and, 6
  variability, 32
Immunological responsiveness, teratocarcinomas stem cells and, 190
Insects, genes for RNA in, 253
Insulin
  actions mediated by cyclic AMP, 285
  assay, 108
  mammary gland differentiation and, 86, 87
    synthesis and, 85
  manufacture, in endocrine pancreas, 107
    in pancreas, 104
Islets of Langerhans, 104
I-somes, DNA, 281
Isotypic variation, immunoglobulin, 32

K

Kidneys, LDH subunits in, 46, 47
Kupffer cells in liver, 158

L

Labelling, nuclear transplantation and, 141
α-Lactalbumin, synthesis, 83
Lactate dehydrogenase
  degradation, 51, 52
  differential gene function in eukaryotes and, 176–181
  experimental systems of differential gene function in eukaryotes and, 41–53
  in lens, 80
  LDH$_a$, distribution, 45, 46
  LDH$_b$, distribution, 45, 46
  LDH$_x$, 42, 43
  structure, 42
  subunit C, 44, 45
  subunit E, 44, 45
β-Lactoglobulin, synthesis, 83
Lamp brush chromosomes, chromomeric chromatin in, 271, 272
Leguminous plants, haemoglobin in, 54
Lens
  fibre, differentiation *in vitro*, 81, 82
  regeneration, 81
Leucocytes, LDH subunits in, 46, 47
Levulinic acid, δ-amino-, production, 64
Lipase
  manufacture in exocrine pancreas, 105
  in pancreas, 104
Lipovitellins, production, 98
Liver
  amphibian, enzyme synthesis in, 163, 164
  differential gene function in eukaryotes and, 158–165
  enzyme accumulation in development of, 159
  enzymes in, hormonal control, 161, 162
  α-foetoprotein in, 164, 165
  inducible enzyme synthesis in, 162, 163
  interaction with oestrogen, egg yolk protein synthesis and, 98
  LDH subunits in, 47
Livetin production, 98
Lungs, LDH subunits in, 46, 47
Luteinizing hormone, actions mediated by cyclic AMP, 285
Lymph nodes, LDH subunits in, 46, 47
Lymphocytes, classes, 40, 41
Lysozyme synthesis, 100

M

Maize
  somatic rearrangement of chromosomal material in, 40
  unstable genetic elements, 275
  unusual genetic elements in, 238–241
Mammals
  embryo, differential gene function in, 184–192
  genes for RNA in, 253
  lactate dehydrogenase in tissues of, 42
  LDH$_a$ in, 44
Mammary glands, differentiation and synthesis, hormonal influence on, 84–86
Melanocyte stimulating hormone, actions mediated by cyclic AMP, 285
Melatonin, actions mediated by cyclic AMP, 285
Merotomy, *Acetabularia*, 208, 210
Methylases, differential gene function in eukaryotes and, 176–181
Mice
  haemoglobins in, ontogeny and, 57
  LDH isozyme patterns in, ontogeny of, 49, 51
Microinjection
  cell contents, 145, 146
  differential gene function in eukaryotes and, 136–151
  mRNA, 146–150
Micromanipulation, nuclear transfer by, 137–140
Milk, production, 83
Milk protein
  experimental systems of differential gene function in eukaryotes, 82–88
  genes, linkage, 87, 88
  genetic variants, 87
Mitochondria
  differential gene function in, 241–249
  DNA, 281–283
  genetic and gene expression, 241–244
Mitosis in plasmodial slime moulds, genetic regulation, 198
Molluscs, tropomyosin A in, 125
Monkeys, LDH distribution in tissues of, 48
Mosses, haploid-diploid differentiation in, 266
Muscle
  cardiac, tissue structure, 118
  differential gene function in eukaryotes and, 117–130
  differentiation, 121
  proteins in, controls of levels of, 127–130
  skeletal, development in embryo, 120–122
    LDH subunits in, 47
    structure, 118–120
  smooth, structure, 118
  striated, structure, 118
Mycoplasma, genes for ribosomal RNA in, 252
Myoglobin
  in muscle cells, 126, 127
    control of level, 128
Myosin
  in muscle cells, 122, 123
    control of level, 127
  synthesis in skeletal muscle in embryo, 120
Myxomycetes
  differential gene function and, 192
  plasmodial, 197–200

N

Negative control, bacterial operons and, 17–19
Neurospora
  chromatin, basic protein in, 167
  differential gene function in, 130–136
  genes, positively controlled, 133
  haemoglobin in, 54
  haploid content, 131
  poky mutant, 244
  RNA polymerase, 175
Nicotiana glauca × *Nicotiana langsdorffi*, 236
Nitrate reductase
  in *Aspergillus*, gene clustering, 135
    genetic control, 133
Nitrite reductase in *Aspergillus*, genetic control, 133
Normoblast, 61
Nuclear membranes in prokaryotic organizations, 15
Nuclear transfer by micromanipulation, 137–140
Nuclear transplantation
  in *Acetabularia*, 209, 210

  differential gene function in eukaryotes and, 136–151
  labelled molecules and, 141
Nucleic acid
  replication in viruses, 22
  single stranded, hybridization, 8
Nucleotidyl transferase, differential gene function in eukaryotes and, 176–181
Nucleus, screening of RNA transcripts by, 10

O

Oestrogen, interaction with liver, egg yolk protein synthesis and, 98, 99
Ontogeny
  crystallins and, 78
  haemoglobins and, 55, 57, 58
  LDH patterns and, 51
Operons
  in bacteria, 17–19
  in eukaryotes, 272
Ovalbumin
  specific mRNA for, 261
  synthesis, 100
Ovaries, uptake of vitellogenin by, 99, 100
Ovomucoid synthesis, 100

P

Pancreas
  differential gene function in eukaryotes and, 104–111
  differentiation, 106
  endocrine, differential gene function in eukaryotes, 107–111
  exocrine, differential gene function in eukaryotes, 105–107
  LDH subunits in, 46, 47
Paramecium
  cortical inheritance, 200–202
  cortical patterns, 202–205
  cortical mutants, 204
  haemoglobin in, 54
Parascaris, differentiation by chromosome reduction, 267
Parascaris equorum, gene elimination in, 3
Parathyroid hormones, action mediated by cyclic AMP, 285
Petite mutant in yeast, 244–246
Phase changes in *Hedera helix*, 236–238
Phosphorylase
  in muscle cells, 125
    control of level, 129
Phosvitin, production, 98
Physarum
  actin in, 124
  differential gene function and, 192
  differentiation, 198
  enzyme synthesis in, 198
  life cycle, 199
  myosin in, 123
Phytohaemagluttinin, gene expression and, 288
Pigeons
  lactate dehydrogenase in, 43
  LDH_c in, 44
Placenta, LDH subunits in, 46, 47
Plant growth substances, gene expression and, 286, 287
Plants, hormones, gene expression and, 286, 287
Plasmids, bacterial, 28
Poly A
  attachment to Hn RNA, 10
  attachment to mRNA, 264
Polychromatophilic erythroblast, 61
Polymerization, proteins, gene expression and, 12
Polypeptides
  gene expression in animals and, 284
  release from ribosomes, 12
Polyploidy, silk glands, 89
Positive control, bacterial operons, 19–21
Post-transcriptional control of gene expression, cytoplasm and, 279–283
Procarboxypeptidase A, manufacture in exocrine pancreas, 105
Procarboxypeptidase B, manufacture in exocrine pancreas, 105
Proelastase, manufacture in exocrine pancreas, 105

Proerythroblast, 61
Proinsulin, 108
Prokaryotes
  gene expression in, 14–29
  genetic organization in, 15, 16
  genome, structure, 16
  ribosomal RNA in, 250
Prolactin
  casein synthesis and, 87
  erythropoiesis and, 71
  mammary gland differentiation synthesis and, 85
Prostaglandins, actions mediated by cyclic AMP, 285
Protamine in sperm, 166
Proteases, differential gene function in eukaryotes and, 176–181
Proteins
  cortical, in *Paramecium*, 200–202
  differential gene function in eukaryotes, experimental systems involving one type of, 30–103
  genome associated, in fungal cells, 131
  mRNA complexes, 10, 11
  in muscles, control of level of, 127–130
  post-translational modification, 12
  storage or degradation, 13
  synthesis as expression of genes, 1
  synthesis in *Acetabularia*, 211–213
  temporal regulation of synthesis in bacteria, 22
Proteus, R factor in, 28
Protozoa, cortical inheritance, 200–205

R

Rabbits, LDH distribution in tissues of, 48
Rana catesbiana
  haemoglobins in, 55
    ontogeny and, 58
Rana pipiens
  LDH isoenzyme patterns in, 50
  nuclear transfer in, by micromanipulation, 137
Rats
  LDH distribution in tissues of, 48
  LDH_5, half life, 52
Regeneration
  in explanted *Hydra* tissue, 217–219
  lens, 81
Regulation
  genes, general concepts, 266–288
  of gene expression, after translation, 280
Reticulocytes, 61
R Factors, bacterial, 28
Rhyncosciara, 9S histone mRNA from, 171
Ribonuclease manufacture in exocrine pancreas, 105
Ribosomes
  function, 251, 252
  release of polypeptide chains from, 12
  RNA in, 250–257
  in translation of mRNA, 11
Rifampicin, transcription and, 8
RNA
  gene expression and, 250–265
  heterogeneous nuclear, gene expression, 258–261
    Hn, attachment of poly A sequences to, 10
    size reduction of, 10
  messenger, calf lens crystallin, translation of injected, 147
    crystallin synthesis and, 79, 80
    EMC virus, translation of injected, 147
    fibroin, isolation, 91, 92
    gene expression, 261–265
    globin, translation of injected, 147
    haemoglobin synthesis and, 67–70
    half life, 265
      in eukaryotic cells, 215
    honeybee promellitin, translation of injected, 147
    lifespan and degradation, 11, 12
    microinjection, 146–150
    milk proteins, 87
    mouse collagen, translation of injected, 147

    mouse myeloma light chain, translation of injected, 147
    protein complexes, 10, 11
    ribosomal translation, 11
    synthesis, 262, 263
    trout testis protamine, translation of injected, 147
  utilization in cytoplasm, 264, 265
  nuclear, mRNA, 65, 66
  ribosomal, 250–257
    amplification and magnification of genes for, 255–257
    arrangement and expression of genes for, 252–255
    genes, repeated sequences, 270
    haemoglobin synthesis and, 67
    synthesis, 250, 251
  synthesis in *Acetabularia,* 213, 214
  transcripts, nucleus screening of, 10
  transfer, availability, gene expression and, 12
    gene expression and, 257, 258
    genes, repeated sequences, 270
    haemoglobin synthesis and, 66, 67
RNA polymerase
  differential gene function in eukaryotes and, 174–176
  specificity and availability, 7

S

Saccharomyces, haemoglobin in, 54
Saccharomyces cerevisae
  enzyme synthesis in, 116
  gene clustering in, 133, 134
  synchronous culture, 112
Salmonella
  phase variation genetics, allelic exclusion in, 37
  somatic rearrangement of chromosomal material in, 40
Salmonella typhimurium, histidine synthesis, operons for, 17
Schizosaccharomyces pombe
  sucrase and maltase induction in, 115
  synchronous culture, 112
Sciara, differentiation by chromosome reduction, 267
Sea urchin
  polysomes, 9S histone mRNA from, 171
  sperm, flagellae, actin from, 124
Sendai virus, cell fusion and, 141
Sequential control of gene expression in prokaryotes, 21–23
Sex factor F, 28
Sickle cell anaemia
  haemoglobin in, 57
  haemoglobin structure and, 56
Silk, production, 88
Silk glands, polyploidy, 89
Slime moulds
  cellular, differential gene function and, 192–197
  chromatin, basic protein in, 167
  differential gene function and, 192–200
  LDH in, 51
  plasmodial, 197–200
Somatic mutation
  idiotypic variation in antibody structure and, 38, 39
  theory, antibody diversity, 34
Spermatocytes, LDH$_c$ in, 44
Spherulation of *Physarum,* 199
Spleen, LDH subunits in, 46, 47
Sporulation
  bacteria, post-transcriptional control and, 27
  in *Physarum,* 199
Stentor
  cortical inheritance, 200
    cortical patterns, 202–205
  cortical mutants, 204
Steroids
  gene expression in animals and, 283, 284
  regulation of egg white protein synthesis by, 101, 102
Synchronization, yeast cell cultures, 112
Synchrony of multinucleate plasmodium, 198

T

T cells, 40
Teratocarcinomas, 189

Teratomas from embryonic cells, 188–190
Tertiary structure, base sequence recognition by, 24
Tetrahymena
  actin in, 124
  haemoglobin in, 54
Tetrahymena pyriformis, myosin in, 123
Thymus, LDH subunits in, 46, 47
Thyroid, LDH subunits in, 46, 47
Thyroid stimulating hormone, actions mediated by cyclic AMP, 285
Thyroxine
  erythropoiesis and, 71
  liver enzyme control by, 161
Tobacco plant, callus tissue, single cell culture, 234
Tonomyosin, 118
Transcription
  anatagonists, 7, 8
  control in prokaryotes, 16, 17
  fibroin synthesis control and, 89–91
  genes, control, 3–7
  gene expression control after, 24–27
  haemoglobin synthesis and, 59
  haemoglobin synthesis control by globin after, 70
  in prokaryotes, 15
  regulation, experimental systems, 7–9
  of single genes, controlled, 272–276
Transdetermination
  in *Drosophila* homeotic mutants, 228
  in *Drosophila* imaginal discs, 231, 232
Translation
  fibroin synthesis control and, 89–91
  gene expression regulation after, 280
  mRNA, ribosomal, 11
  in prokaryotes, 15
Translocation, antibody diverity and, 39, 40
Translocon, immunoglobulin genetics and, 39
Transplants, organs, cartilage differentiation in, 155
Tropomyosin in muscle cells, 122–125
Troponin in muscle cells, 122, 123, 125
Trypsin inhibitor, manufacture in exocrine pancreas, 105
Trypsinogen
  in pancreas, 104
  manufacture in exocrine pancreas, 105
Twins, mammalian monozygotic, differing phenotypes of, 202
Tyrosine, tRNA, in *Escherichia coli,* 257
Tyrosine amino transferase in liver, hormonal control, 161, 162

U

Uridine, 5-bromo-2′-deoxy-
  cartilage differentiation and, 155–157
  gene expression and, 276
Uterus, LDH subunits in, 47

V

Vasopressin, actions mediated by cyclic AMP, 285
Vertebrates, erythroid cells in, 60
Virion polymerases, 21
Viruses
  bacterial, transcription, mechanisms, 23
  gene nature in, 16
  genetic organization in, 15
  sequential control of gene expression in, 21–23
  translocon hypothesis and, 40
  tumour, translation of injected, 147
Vitellogenin, 98
  uptake by ovaries, 99, 100

X

Xanthine dehydrogenase II, in *Aspergillus nidulans,* negative control, 132
Xenopus
  anaemic, blood, 62
  egg yolk protein synthesis in, 98

- globin mRNA in, haemoglobin synthesis and, 68
- 9S histone mRNA from, 171
- nuclear transfer in, by micromanipulation, 138
- oocytes, mRNA injection into, 146
- preservation of allotypic identity in, 38
- RNA polymerase, 175
- tadpole haemoglobin production in adults, 59

Xenopus laevis
- gene amplification in, 271
- genes for RNA, 253
- haemoglobin in, 55
- haemoglobins in, ontogeny and, 58
- lens regeneration in, 81
- oocytes, gene copies of rRNA in, 255

Xenopus laevis × *mulleri* hybrids
- ribosomal RNA synthesis in, 6

Xenotypic variation, immunoglobulin, 32

Y

Y Chromosomes, 17

Yeast
- cell cycle, enzyme synthesis during, 111–117
- genes for RNA in, 253
- petite mutant, 244–246

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18

Control of Gene Expression

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